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"Dysfunctional PAR_1 signaling in the malignant mesothelioma cell line, NCI-H28"

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INTRODUCTION

1 PROTEASE ACTIVATED RECEPTOR

Seven transmembrane G protein coupled receptors (GPCRs) are the largest group of membrane receptors in mammalian systems and many of them are target of drugs which are currently in clinical use. Among this receptor family, protease-activated receptors (PARs) are a subset which have a unique mechanism of activation. In fact, rather than being activated by simple ligand occupancy, they are activated enzymatically through proteolysis by enzymes of the serine protease family (Macfarlane *et al*., 2001). The proteolytic cleavage occurs at specific sites within their N-terminal region, thereby exposing novel N-termini, and the 'tethered ligand' then folds back onto the extracellular loop II of the receptor, resulting in activation.

PARs are expressed in various tissues and cell types, such as cardiomyocytes, platelets, fibroblasts, endothelial cells and cells of the gastrointestinal tract and lung. Activation of these receptor types is involved in various cellular responses associated with haemostasis, inflammation and cell proliferation, thus making them attractive targets for the therapeutic treatment of several diseases including thrombosis, atherosclerosis, cancer metastasis and inflammation.

There are four PARs encoded by distinct genes in the mammalian genome. The prototype of this GPCR subfamily is PAR_1 which was discovered in 1991 (Vu *et al.,* 1991; Rasmussen *et al.,* 1991) and transmits cellular response to thrombin, the main effector protease of the coagulation cascade. Soon after, other three members of the subfamily were identified which include PAR_2 (Nystedt *et al.,* 1995), the trypsin-activated receptor, and two other thrombin-activated receptors, PAR_3 (Ishihara *et al.,* 1997) and PAR_4 (Xu *et al.,* 1998). Whereas cleaved PAR_4 is able to activate diverse signaling pathways the ability of activated PAR_3 to signal on its own is not completely clear. PAR_1 seems to act as a cofactor for

thrombin-mediated activation of PAR_4 (Nakanishi-Matsui *et al.,* 2000) and may also signal as part of a PAR_1-PAR_3 heterodimer (McLaughlin *et al.,* 2007). Other proteases besides trypsin for PAR_2 and thrombin and trypsin for PAR_1 and PAR_4 can activate these receptors (Soh *et al.,* 2010) as well as synthetic peptides that mimic the first six amino acids of the newly formed N-terminus and act as soluble ligands in the absence of receptor proteolysis. Some PAR agonist peptides (PAR-APs) activate more than one PAR at concentrations in the micromolar range, as compared with nanomolar potencies of the proteases themselves.

1.1 Mechanism of activation by cell-surface proteolysis

1.1.1 Proteases cleavage and exposure of tethered ligand domains

The general mechanism by which proteases cleave and activate PARs is the same: proteases cleave at specific sites within the extracellular amino terminus of the receptors. This cleavage exposes a new amino terminus that serves as a tethered ligand domain, which binds to conserved regions in the second extracellular loop of the cleaved receptor, resulting in the initiation of signal transduction. There is no known function of the amino-terminal fragment of the receptor that is removed by proteolysis. The mechanism by which thrombin activates PAR_1 has been investigated in detail. Thrombin cleaves PAR_1 at $R^{41}\downarrow S^{42}$FLLRN in the N-terminal region, to expose the tethered ligand NH_2-SFLLRN, which binds and activates the cleaved receptor, resulting in signal transduction. Several observation support this mechanism of activation. Mutation of the cleavage site prevents thrombin cleavage and signalling, indicating the importance of this site for PAR_1 activation (Vu *et al.*, 1991). A synthetic peptide (S^{42}FLLRN) that mimics the tethered ligand domain directly activates intact PAR_1, without the requirement for hydrolysis by thrombin. Such synthetic agonists are useful

tools for investigating PAR functions. Similar observations indicate that trypsin cleaves PAR_2 at $R^{34}\downarrow S^{35}$LIGKV to reveal the amino-terminal tethered ligand SLIGKV in humans (Nystedt *et al.*, 1995). Synthetic peptides corresponding to the tethered ligand domain SLIGKV activate PAR_2 without the need for receptor cleavage. PAR_3 is cleaved by thrombin at $K^{38}\downarrow F^{39}$RGAP and mutation of the cleavage site to one that would be resistant to thrombin prevents its activation (Ishihara *et al.*, 1997). This cleavage exposes a new amino terminus (TFRGAP) that may interact with the receptor as a tethered ligand. However, in marked contrast to PAR_1, PAR_2 and PAR_4, synthetic peptides corresponding to this putative tethered ligand do not activate PAR_3. The reason for this discrepancy is unknown, although differences in affinity, steric hindrances and the possibility that cleavage releases conformation of the receptor constrained by the uncleaved amino terminal region could explain these unexpected results. Another unexpected observation is that mouse PAR_3 is unable to signal when expressed alone, without other PARs (kahn *et al.*, 1998). Thrombin and trypsin cleave PAR_4 at $R^{47}\downarrow G^{48}$YPGQV and peptides corresponding to the tethered ligand domain GYPGQV can directly activate PAR_4 (kahn *et al.*, 1998). Mutation of the cleavage site prevents activation by thrombin and trypsin but not by the synthetic peptides, which confirms the importance of the proteolytic cleavage for receptor activation.

1.1.2 Facilitation of PARs cleavage and activation by protease binding

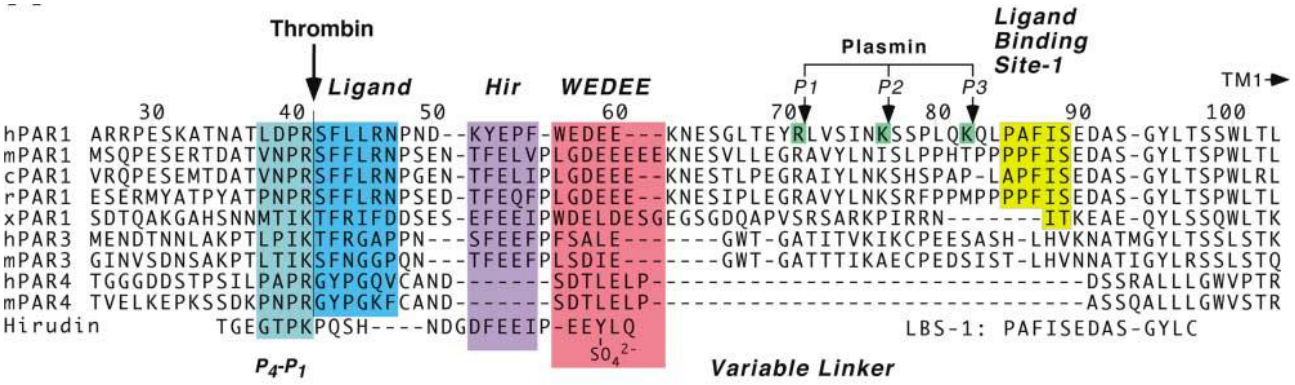
Thrombin can activate PAR_1 and PAR_3, by a two-step mechanism: first the protease binds and then it cleaves the receptor. The process of binding and activation has been most thoroughly studied for thrombin and PAR_1 (Vu *et al.*, 1991). In addition to containing the tethered ligand, the PAR_1 exodomain also harbors a hirudin-like (Hir) sequence element,

$K^{51}YEPF^{55}$, that is essential for high-affinity interactions with thrombin. This charged domain is distal to the thrombin cleavage site and binds to an anion binding site on thrombin, thereby temporarily concentrating the protease at the surface of the receptor. This negatively charged region of PAR_1 resembles a domain of the leech anticoagulant hirudin, which inhibits thrombin by binding its anion site. The importance of the hirudin-like domain is emphasized by the finding that its deletion markedly diminishes the capacity of thrombin to activate PAR_1 whereas substitution of this region with the corresponding domain of hirudin allows a full recovery of activity. Steady-state kinetic studies using soluble full-length PAR_1 exodomains showed that the initial binding of the Hir sequence of PAR_1 with exosite I is essential for rapid association to thrombin. In a second and rate-limiting step, the exosite I-bound Hir motif facilitates the productive interaction of the PAR_1 cleavage sequence to the active site of thrombin. The subsequent irreversible steps of peptide bond cleavage are rapid and allosterically enhanced by the presence of the docked Hir sequence. Following cleavage, thrombin remains associated with the C-terminal portion of the PAR1 exodomain via this high-affinity Hir sequence. Complementary studies with an array of thrombin active site and exosite mutants further demonstrated the importance of the thrombin exosite I for efficient binding and cleavage of PAR_1 peptides. Docking studies of the PAR_1 exodomain Hir motif to exosite I of thrombin reveals that the active site of thrombin is readily accessible to another large macromolecular substrate. This supports the notion that an additional role of the PAR_1 Hir sequence is to tether thrombin to the platelet surface and assist in the cleavage of other nearby platelet receptors that lack a high-affinity Hir-like sequence.

Adjacent to the Hir-motif is an anionic E57DEE60 cross-cluster that splays outward from the exodomain where it is available for electrostatic interactions with the positively charged thrombin exosite-1. A hydrophobic scaffolding residue, Trp^{56}, anchors both the adjacent Hir and EEDE motifs by making numerous contacts with Pro^{54}, Phe^{55}, Glu^{57}, Asp^{58},

Leu[66] and Thr[67]. Kinetic analyses of soluble PAR_1 exodomains showed that the presence of Trp[56] enhances the affinity of the adjacent Hir sequence for exosite I of thrombin by 11-fold.

The N-terminal tethered ligand forms an intramolecular complex with a region located in the C terminus of the PAR_1 exodomain that was confirmed to be an authentic ligand binding site LBS-1 ($P^{85}AFIS^{89}$) in full-length receptor.



PAR_3 also contains a hirudine-like site (FEEFP) that is distal to the thrombin cleavage site, which interacts with thrombin (Ishihara *et al.*, 1997). PAR_4, in contrast to the other thrombin receptors, lacks a hidurin-like binding site for thrombin (Xu *et al.*, 1998). Protease binding site have not been identified for PAR_2.

1.1.3 Functional interactions between PARs

A common theme of signalling by GPCRs is that there are frequently several different receptors for a single ligand and often several ligands for one receptor. Thus thrombin can activate PAR_1, PAR_3 and PAR_4, with different potencies and trypsin, tryptase and certain coagulant factors can activate PAR_2. This complexity becomes particularly interesting because PARs are frequently coexpressed and interactions between receptors in the same cell

can have important functional consequences. Human platelets express two thrombin receptors, PAR_1 and PAR_4. There are marked differences in the mechanisms of activation and inactivation of these receptors that have important consequences for thrombin signalling to platelets. As previously explained, PAR_1, in contrast to PAR_4, responds to low concentrations of thrombin and its responses are rapidly shut-off, probably as a result of phosphorylation of residues in the carboxy terminus and uncoupling from G proteins while PAR_4 responses are sustained and desensitize slowly (Kahn *et al.*, 1999). The coexpression of two receptors with different potencies and kinetics of desensitization may have important functional consequences. Thus, in human platelets PAR_1 mediates rapid and transient increases in intracellular calcium concentration ($[Ca^{2+}]_i$) to low concentrations of thrombin whereas PAR_4 mediates delayed and sustained increases in $[Ca^{2+}]_i$ to higher thrombin concentrations (Covic *et al.*, 2000). This prolonged signal is important for the late phases of platelet aggregation. Similar dual receptor system probably exist on other cells with important functional consequences.

In some instances, protease binding to one receptor can facilitate cleavage of another receptor that is expressed on the same cell. This appear to be the situation for PAR_3 and PAR_4 on murine platelets. PAR_3 acts as a cofactor for PAR_4 in murine platelets where it facilitates activation of PAR_4 by low concentrations of thrombin. The hirudin-like site of PAR_3 binds and concentrates thrombin at the cell surface, and thereby promotes thrombin cleavage of PAR_4. At high concentrations, thrombin can directly cleave and activate PAR_4, even though it lacks a thrombin binding domain.

GPCRs can form homodimers and heterodimers with important consequences for signal transduction. Although the principal mechanism of PAR activation is intramolecular, there are several examples of intermolecular interactions between different PAR molecules. Intermolecular signalling, by which a cleaved receptor can activate an uncleaved receptor,

was first demonstrated for PAR_1 (Chen *et al.*, 1994) when in a reconstituted system was shown that is possible for the tethered ligand of cleaved PAR_1 to activate an uncleaved receptor. There is also evidence of intermolecular signalling between different PARs. Peptides corresponding to the tethered ligand of PAR_1 (SFLLRN) can also activate PAR_2, but not vice versa (Blackhart *et al.*, 1996). Several results suggest that the tethered ligand domain of PAR_1 can interact with uncleaved PAR_2 to transduce the signal (O'Brien *et al.*, 2000). Recently it has been demonstrated that PAR_1/PAR_3 heterodimers are formed constitutively in endothelial cells and the activated dimeric complex induces distinct signalling involving the selective coupling to $G\alpha_{13}$ as compared with the PAR_1/PAR_1 homodimer. The ability of PAR_1 to form heterodimers with PAR_3 indicates that this heterodimer is formed under physiological conditions and could be implicated in endothelial barrier dysfunctions (McLaughlin *et al.*, 2007). This novel form of intermolecular signalling between different PARs clearly requires the close association of receptors at the cell surface, which could be influenced by levels of expression or by anchoring proteins that may affect mobility of receptors in the membrane.

1.2 Signal transduction from protease activated receptors

1.2.1 Structure-activity relations of tethered ligand domains

The observation that synthetic peptides corresponding to the tethered ligand domain of PAR_1 are agonists for the receptor without the need for proteolysis had three important consequences. First, it enabled the use of syntethic peptides as probes for PAR function thereby avoiding the sole use of proteases, which have many biological effects not related to PARs. These APs are now widely used to investigate the physiological functions of PARs *in*

vitro and *in vivo*. Second, it permitted convenient structure activity studies of the tethered ligand domain through functional analyses of synthetic peptides (Maryanoff *et al.*, 2001). Such studies have provided important information about critical residues of the tethered ligand and facilitated the development of more selective agonists (Hollenberg *et al.*, 1997). Third, analogues of the APs have been used as templates for the development of antagonists of PAR_1 (Andreade-Gordon *et al.*, 1999). However, there are certain limitations to the use of APs as PAR agonists. Unfortunately, APs are weak agonists compared with proteases, often requiring concentrations in excess of 1 μM to activate most PARs. The low potency of APs is mostly attributed to the inefficient presentation of these soluble peptides to the binding domains of the receptor compared with the tethered peptide. In addition, these peptides are readily inactivated by proteolysis. Because APs are used at such high concentrations, they may have nonspecific effects that are unrelated to PAR activation. The use of control peptides, such as inactive scrambled peptides or reversed sequences, is thus essential, although these too can have activity. In addition, APs are not always specific for a particular PAR; for example, the PAR_1-AP SFLLRN also activates PAR_2 (Blackhart *et al.*, 1996).

Replacement of Ser^1 with Thr^1 (corresponding to the sequence in *Xenopus* PAR_1) in PAR_1-AP generates an agonist TFLLRN that is selective for PAR_1 and that does not activate PAR_2.

1.2.2 Activation of multiple signaling cascades

Activated PAR_1 and PAR_2 couple to multiple heterotrimeric G-protein subtypes including G_i, G_q and $G_{12/13}$ (Coughlin, 2005; Soh *et al.*, 2010), whereas PAR_4 couples to G_q and $G_{12/13}$ activation. In doing so, PAR_1 and PAR_2 also activate numerous effector pathways

in most cell types including mitogen-activated protein kinases (MAPKs), various Rho kinase and phospholipase C (PLC) isoforms, inositol lipid signaling and mobilization of intracellular Ca^{2+} (Macfarlane *et al*., 2001). $G\alpha_q$ stimulates phospholipase C to produce inositol trisphosphate (IP_3), which mobilizes intracellular Ca^{2+}, and diacylglycerol (DAG) activating protein kinase C (PKC). Together, Ca^{2+} and PKC activate numerous downstream pathways, including Ca^{2+}-regulated protein kinases and mitogen-activated protein (MAP) kinases. $G\alpha_{12/13}$ plays a major role in the control of cell shape and migration through interaction with Rho guanine-nucleotide exchange factors (Rho-GEF). $G\alpha_i$ proteins inhibit adenylyl cyclase and suppress formation of cAMP. $G\beta\gamma$ subunits of heterotrimeric G proteins couple PAR_1 to many other pathways, notably activation of phosphatidylinositol (PI) 3-kinase, which promotes Ca^{2+} release. PI 3-kinase thus links PAR_1 to changes in cytoskeletal structure, cell motility, survival and mitogenesis.

There has been considerable interest in understanding the mechanism by which PAR_1 couples to the MAP kinase cascades given the important mitogenic role of thrombin. Many data about PAR_1-mediated ERK1/2 activation derive from studies in astrocytes since PAR_1 stimulate astrocytes proliferation both *in vitro* and *in vivo*. PAR_1 induces robust ERK1/2 activation, an important mediator of astrocyte proliferation. In contrast to transient ERK1/2 activation induced by many stimuli, activated PAR_1 causes sustained ERK1/2 signaling in astrocytes (Nicole *et al.*, 2005; Suo *et al.*, 2003). PAR_1 stimulation causes the activation of transcriptional factor nuclear factor κB in endothelial cells.

PARs, like other GPCRs, can exhibit biased signaling (Rajagopal *et al.*, 2011; Kenakin and Miller, 2010). For example, the activation of PAR_1 with a peptide agonist can cause signaling preferentially via G_q, whereas thrombin-triggered PAR_1 signaling is preferentially coupled to $G_{12/13}$ (McLaughlin *et al.*, 2005). Furthermore signaling via PAR_1 and PAR_2 can differ depending on receptor location with caveole localization of PAR_1 being required for

protease-selective signaling (Russo *et al.,* 2009). Finally, it is important to mention, that activated PARs also interact with various adaptor proteins that facilitate signal transduction independent of heterotrimeric G protein coupling. Among these proteins, the multifaceted β-arrestins play a major role controlling magnitude and duration of mainly PAR_2- but also PAR_1-mediated G protein signaling as well as PAR_2 signaling to non-G protein effectors (Chen *et al.,* 2004; Soh *et al.,* 2010).

1.3 Termination of the signal

1.3.1 Disablement of PARs by cell-surface proteolysis

Proteases that remove or destroy the tethered ligand or cleave the binding domain in the extracellular loop II would generate receptors that are unresponsive to activating proteases. Many proteases can disable PARs in this manner. Disabling proteases may serve to dampen signalling by activating proteases and could thereby be an additional mechanism for terminating protease signalling. A portion of proteolytically activated PAR_1 can recycle to the cell surface in some cell types, although it is usually targeted for degradation. Proteolytically activated and recycled PAR_1 can continue to signal at the cell surface (Trejo *et al.*, 1998). In this case, proteases that cleave or remove the exposed tethered ligand would arrest signalling. Proteases from inflammatory cells, including neutrophils and mast cells, can cleave and disable PARs. Neutrophil cathepsin G, elastase and protease 3 cleave PAR_1 removing the activation site and thereby abolish thrombin signalling (Renesto *et al.,* 1997). PAR_1-AP still signals to cells exposed to these proteases, suggesting that the binding domain is preserved. Elastase and cathepsin G cleave PAR_2 and PAR_3 in transfected cells removing the amino-terminal epitopes and thereby generating receptors that are unresponsive to trypsin

and thrombin, respectively. However, PAR_2 maintains its responsiveness to PAR_2-AP (Dulon *et al.*, 2003). Interestingly, this process of inactivation occurs without the loss of binding of monoclonal antibodies that recognize sites flanking the tethered ligand domain suggesting inactivation does not involve removal of the tethered ligand. The mechanism of this inactivation is unknown but could involve cleavage of tethered ligand binding domains. Some proteases can cleave PARs at several sites, including activation and disabling sites, and the net result depends on the efficiency of cleavage at different locations. Although trypsin has been reported to activate PAR_1, it also efficiently cleaves PAR_1 at distal sites that would remove the tethered ligand domain. Indeed, in endothelial cells, trypsin inactivates PAR_1, generating a receptor that is unresponsive to thrombin (Nakayama *et al.*, 2003). In addition to cleavage at the activation site (Arg^{34}-Ser^{35}), tryptase also cleaves PAR_2 at the Lys^{41}-Val^{42} site, which could inactivate the receptor. In the case of tryptase, the activating cleavage is more important since tryptase activates PAR_2. The consequences of exposing cells to proteases depend on the repertoire of PARs expressed by a cell and whether the proteases activate or disable particular receptors. For example, cathepsin G disables PAR_1 but activates PAR_4. Thus in human platelets, which express PAR_1 and PAR_4, cathepsin G can induce aggregation through PAR_4 even though it disables PAR_1 and thereby prevents signalling by low concentrations of thrombin. In contrast, in fibroblasts and endothelial cells that do not express PAR_4, cathepsin G abolishes thrombin signalling through PAR_1. In addition, proteolysis can impair the ability of PAR_3 to act as a cofactor for PAR_4 (Cumashi *et al.*, 2001). In murine platelets, PAR_3 binds thrombin but does not signal. Instead, it concentrates thrombin in the vicinity of PAR_4 and thus serves as cofactor for PAR_4 signaling in response to low concentrations of thrombin. Cathepsin G does not cause aggregation of murine platelets but prevents aggregation to low concentrations of thrombin, indicating that cathepsin G abolishes this cofactor role of PAR_3 (Ossovskaya and Bunnett, 2004).

1.3.2 Receptor desensitization

Proteases activate PARs by an irreversible mechanism: cleavage exposes the tethered ligand domain that is always available to interact with the cleaved receptor. This activation would result in prolonged signalling unless there were efficient mechanisms to attenuate the response. The principal mechanism that terminates signalling by PARs is broadly similar to the classical pathway of desensitization that has been described in detail for many other GPCRs, in particular rhodopsin and the β_2-adrenergic receptor (Bohm *et al.*, 1997; Luttrell and Lefkowitz, 2002).

Ligand occupation of the GPCR induces the translocation of members of the family of G protein receptor kinases (GRKs) from the cytosol to the activated receptor at the cell surface. GRKs are serine-threonine kinases that phosphorylate activated GPCRs usually within the carboxy terminus or third intracellular loop. Phosphorylation triggers the membrane translocation of arrestins, which interact with the phosphorylated transduction. Additional mechanism may involve phosphorylation by second messenger kinases, which also terminate signalling. However, there are still many critical aspects of GPCRs desensitization, including PARs, which are unexplored. Several observations suggest that modifications of the tethered ligand domain of cleaved PAR_1 may also contribute to desensitization. One mechanism may involve cleavage of the tethered ligand domain. The hypothesis is that after activation with AP, the tethered ligand domain is proteolytically destroyed, rendering the receptor unresponsive to thrombin but not AP. PAR_4 has slow kinetics of desensitization that permits it to signal in a sustained fashion in human platelets. However, mechanisms of desensitization vary between different PARs, probably due to structural differences, especially in the intracellular loop III and carboxy terminus (Traynelis and Trejo, 2007).

1.3.3 Downregulation by intracellular proteases

In addition to processes that regulate coupling of receptors to signalling pathways, cells also determine their responsiveness to agonists by regulating the levels of receptors that are expressed at the plasma membrane and which are thus accessible to agonists in the extracellular fluid. The level of expression of receptors at the cell surface is a balance between removal by endocytosis and replenishment by recycling or mobilization of intracellular pools. Many receptors internalize after binding agonists. However, the fate of endocytosed receptors depends on postendocytic sorting, which varies from receptor to receptor. At one extreme, some GPCRs that are activated in a reversible fashion by agonist binding, for instance the neurokinin-1 receptor, efficiently recycle to the plasma membrane to be reused by the cell (Defea *et al.*, 2000). At the other extreme, receptors such as the PARs, which, once cleaved, cannot be reused by the cell, are destined for intracellular degradation, which irrevocably terminates signalling. For these receptors, recovery requires synthesis or mobilization of new receptors. Of course, all surface receptors have a finite life span and even recycling receptors are downregulated by intracellular degradation after long-term stimulation.

The process of receptor downregulation is of considerable importance because defects can result in exaggerated signalling and abnormal phenotypes, for example, transformation in the case of EGF receptors that are not downregulated. PARs are an ideal model to study these postendocytic sorting events since they are invariably targeted for degradation after activation. The molecular mechanism and pathways of agonist-induced trafficking of PARs vary from receptor to receptor and in different cells. The newly synthesized PAR_1 contains a signal peptide which targets the receptor to the plasma membrane.

PAR_1 expressed on the plasma membrane is then subjected to two distinct modes of internalization: the constitutive internalization and the agonist-triggered internalization. The stimulation of PAR_1 with either thrombin or agonist peptides triggers the internalization of PAR_1. The internalized receptors are mainly targeted to lysosomal degradation and partly recycled to the plasma membrane (Trejo, 2003). Internalization and lysosomal sorting of proteolytically activated PAR_1 is thought to prevent the receptor from returning to the cell surface with its tethered ligand intact and continuing to signal (Trejo and Coughlin, 1999). Proteolysis or truncation of the tethered ligand may also occur to prevent further PAR_1 signaling. The full recovery of the responsiveness to protease activation requires either de novo synthesis or the recruitment of intact receptors from the intracellular pool. The importance of these mechanisms depends on whether cells possess prominent intracellular stores of PAR_1 in the Golgi apparatus. Platelets, which lack both the ability to synthesize new receptors and prominent intracellular pools, are unable to repopulate the plasma membrane with new receptors after thrombin exposure and thus do not recover responsiveness to thrombin. Because platelets are only required to respond once to thrombin, by aggregation, the lack of a robust system of resensitization does not impair their function. Moreover, human platelets also express PAR_4, which facilitates prolonged signalling by thrombin. Similar mechanisms account for the resensitization of PAR_2 suggesting that these are widespread mechanisms (Hamilton *et al.*, 1999).

Constitutive internalization takes place under resting conditions without any receptor stimulation and requires residues of the C-terminus of PAR_1, which are distinct from those required for agonist-triggered internalization. The cells expressing mutant PAR_1, which has been shown to be defective in constitutive internalization but not in agonist-triggered internalization, are missing the intracellular pool (Shapiro *et al.*, 1998). The nascent PAR_1 is thus considered to target first the plasma membrane and then be internalized into the

intracellular pool. Next, the PAR_1 cycles between the plasma membrane and the intracellular pool under resting conditions. Therefore, the major steps that regulate the level of PAR_1 expressed on the cell surface are transcription, constitutive and agonist-triggered internalization, the recruitment of intact receptors from the intracellular pool and lysosomal degradation (Hirano *et al.*, 2005).

PAR_1 and PAR_2 are rapidly and extensively internalized after activation and the initial accumulation of the receptors into coated pits suggests that internalization proceeds by a clathrin-mediated process. β-arrestins play a major role in endocytosis of many GPCRs by serving as adaptor proteins that link GRK-phosphorylated receptors to clathrin and clathrin adaptor protein-2 (AP2). However, β-arrestin play no role in stimulated endocytosis of PAR_1 while it is required for PAR_2 internalization. Although the molecular mechanisms that target PAR_2 for degradation are unknown, PAR_2 is extensively ubiquitinated after activation and ubiquitination of some other GPCRs is a prerequisite for degradation (Traynelis and Trejo, 2007).

1.4 Physiology and pathophysiology of Protease Activated Receptors

PARs have multiple roles in many physiological and pathological events involving different tissues and organs such as the cardiovascular, musculoskeletal, gastrointestinal, respiratory and central nervous system (CNS) (Ramachandran *et al.*, 2012). In addition, coagulant protease and PARs have been implicate in several types of malignant cancer. PAR_1 is overexpressed in aggressive melanoma (Tellez and Bar-Eli, 2003), colon cancer (Darmoul *et al.*, 2003), prostate cancer (Chay *et al.*, 2002) and invasive breast cancer (Even-Ram *et al.*, 1998). Since PARs mediate the mitogenic action of both thrombin and trypsin they can

regulate tumor cell growth. Indeed, PAR_1 can promote tumor cell invasion and epithelial cell malignancy (Even-Ram *et al.,* 1998; Even-Ram *et al.,* 2001; Bar-Shavit *et al.,* 2011). The expression of PAR_1 and PAR_2 is also increased in stromal fibroblasts of malignant tissues as compared to normal tissues (D'Andrea *et al.,* 2001). In addition, several different proteases which can potentially activate PAR_1 and/or PAR_2 have been identified in tumors including tissue-derived trypsins, members of the coagulation cascade and matrix metalloprotease-1 (MMP-1) (Arora *et al.,* 2007; Ramachandran *et al.,* 2012). The mitogenic activity induced by PAR_1 or PAR_2 stimulation is associated with prolonged ERK1/2 activation (Arora *et al.,* 2007). Activated PAR_1 also fails to be downregulated in highly invasive breast carcinoma, and consequently PAR_1 persistently activates ERK1/2 and induce cellular proliferation and invasion (Booden *et al.,* 2004). Therefore, increased PAR_1 levels in invasive carcinoma cells may be due, at least partially, to a defective trafficking. Of course, other mechanisms contributing to increased PAR_1 mRNA and protein levels are also possible such as changes in gene transcription (Arora *et al.,* 2007; Bar-Shavit *et al.,* 2011). For example, Tellez *et al.* (2003) have demonstrated an inverse correlation between the expression of activator protein-2α (AP2) and the overexpression of PAR_1 in metastatic melanoma cells.

1.5 Aims of the study

Malignant pleural mesothelioma (MM) is a relatively rare but aggressive neoplasm which is associated with occupational exposure to asbestos. Most patients are diagnosed at a late stage, which makes curative resection difficult. Despite aggressive treatment with radiotherapy and/or chemotherapy the prognosis is poor with a median survival time between 8 and 18 months. MM patients are very susceptible to thromboembolic complications

(Nguyen *et al.*, 2008) which can develop as a consequence of the activating effect of malignant cells on the haemostatic system (Noble and Pasi, 2010).

The aim of this study was to investigate PAR_1 expression levels, signaling and mitogenic effects in nonmalignant mesothelial (Met-5A) and MM cells (NCI-H28). In this MM cell line, a homozygous deletion of the β-catenin gene (CTNNB1) has been demonstrated (Shigemitsu *et al.*, 2001) while thrombomodulin (TM), a natural anticoagulant, appears to be silenced by an epigenetic mechanism (Nocchi *et al.*, 2011). Therefore, we considered quite intriguing to study PAR_1 expression and signaling in this cell line and correlate our findings to the known genetic and epigenetic alterations. Our study indicates that the expression levels of both PAR_1 mRNA and protein are increased in NCI-H28 cells but more important, receptor signaling to down-stream effectors is rather dysfunctional. In fact, the only signaling pathway which is fully maintained is that through G_i proteins while those through $G_{12/13}$ and G_q are either reduced or abolished. Whereas the lack of thrombomodulin on plasmamembrane can alter PAR_1 activation the β-catenin deficiency on the E-cadherin/catenin complexes at cell junctions can interfere with caveolin-1 localization and consequently with PAR_1 localization and proper signaling to G_q and $G_{12/13}$.

MATERIALS AND METHODS

2.1 Materials

Penicillin, streptomycin, hydrocortisone, cAMP, Ro 20-1724, protease inhibitor cocktail, isoproterenol and secondary antibodies were products of Sigma-Aldrich Inc. (St. Louis, MO); while [^{3}H]-cAMP (specific activity 31.0 Ci mmol^{-1}) and enhanced chemiluminescence substrate (Western lightning® Plus-ECL) were from PerkinElmer Inc. (Waltham, MA). HMEC-1 cells were a generous gift of Dr E Ades (Centers for Disease Control, Atlanta, GA). NCI-H28 and Met-5A cells were purchased from LGC Standards s.r.l. (Middlesex, UK). MCDB-131 medium, Medium 199, RPMI-1640, Fetal Bovine Serum (FBS), trypsin-EDTA, epidermal growth factor (EGF), L-glutamine, human recombinant insulin, nitrocellulose membrane, Fluo-3 and Pluronic were purchased from Life Technologies Corporation (Paisley, UK). WST-1 was a product of La Roche (Basel, Switzerland). RhoA activation assay kit was obtained from Cytoskeleton, Inc. (Denver, CO). PAR_1-AP (TFLLR-NH_2), a selective PAR_2-AP (2-furoyl-LIGRLO-NH_2) and SCH79797 were products of Tocris Bioscience (Bristol, UK). GB83 was purchased from Axon Medchem BV (Groningen, The Netherlands). Human thrombin (high activity, ≥2800 NIH U mg^{-1} protein) was a product of Calbiochem-Novabiochem Corp. (San Diego, CA). The RNeasy Mini Kit and SYBR Green PCR Kit were purchased from Qiagen GMbH (Hilden, Germany). The Rev Transcription Kit was a product of New England BioLabs (Ipswich, MA). Primary antibodies were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Other agents and reagents were from standard commercial sources and were of the highest grade available.

2.2 Cell cultures

In the present study human dermal microvascular endothelial cells (HMECs-1) that were transformed using SV-40 were used as reference cells which endogenously express PARs (Asteriti *et al.*, 2012). These cells were used to analyze the differential activation of signaling pathways by different PAR_1-activating peptides (PAR_1-APs) and to test the effects of modulators on signaling.

HMECs-1 were cultured in MCDB-131 medium, supplemented with 5% FBS, 1% penicillin/streptomycin (100 units/ml; 100 µg/ml), hydrocortisone (1 µg/ml), epidermal growth factor (0.01 µg/ml) and L-glutamine (2 mM) in atmosphere of 95% air, 5% CO_2 at 37°C. The cells were seeded at 1 x 10^5 cells/ml in 75 ml cell culture flasks coated with 1% gelatin and subcultured after detachment with 0.05% trypsin, 0.5 mM EDTA. In all experiments, cells were used at passages 16-20.

Malignant mesothelioma cells (NCI-H28) and nonmalignant mesothelial cells (Met-5A) were used to analyze the differential activation of signaling pathways by thrombin and different PAR-activating peptides (PAR-APs).

Met-5A cells were cultured in Medium 199 supplemented with 10% FBS, 1% penicillin/streptomycin (100 units/ml; 100 µg/ml), hydrocortisone (400 nM), epidermal growth factor (3.3 nM) and human recombinant insulin (870 nM) in atmosphere of 95% air, 5% CO2 at 37°C. In all experiments, cells were used at passages 3-9.

NCI-H28 cells were cultured in RPMI-1640 medium, with 10% FBS and 1% penicillin/streptomycin (100 units/ml; 100 µg/ml), in atmosphere of 95% air, 5% CO2 at 37°C. The cells were seeded in 75 ml cell culture flasks and subcultured after detachment with 0.05% trypsin, 0.5 mM EDTA. In all experiments, cells were used at passages 3-15.

2.3 Real time RT-PCR

PCR (polymerase chain reaction) was used to verify the expression of PAR_1 and PAR_2 mRNAs in Met-5A and NCI-H28 cells. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). Cells were lysed and homogenized in the presence of a highly denaturating buffer and the sample was then applied to an RNeasy Mini spin column, where the total RNAs bound to the membrane and contaminants were efficiently washed away. High-quality RNAs were then eluted in water. Six microliters of total RNA for each sample were reverse transcribed to cDNA using a specific Rev Transcription Kit (BioLabs). Afterwards, three microliters cDNA were amplified with SYBR Green by specific oligonucleotide primers for PAR_1 and PAR_2 cDNA sequences, as follow:

PAR_1	**Forward:** 5'-TGC TTC AGT CTG TGC GG-3' **Reverse:** 5'-CTC CAT CAA TAA AAG CAG TCC TCT-3'
PAR_2	**Forward:** 5'-AGC TCT GAG TTT CGA ATC GG-3' **Reverse:** 5'-ACT CCT TTT CCA GTG ACG TG-3'

PAR_1 primers were chosen to amplify a product of the expected size of 206 bp, while for PAR_2 a set of primers to amplify a product of 223 bp was used.

PCR was performed for 35 cycles with the following conditions: after initial Taq activation at 94°C for 5 min, each cycle consisted of denaturing at 95°C for 1 min, annealing at 50°C for 30 sec and extension at 72°C for 1 min, with a final elongation at 72°C for 7 min.

The relative expression of PAR_1 and PAR_2, with β-actin as the reference gene, was determined by using the MiniOpticon™ Real-Time PCR Detection System (BioRad). Data were expressed as expression ratios normalized to β-actin.

2.4 SDS-PAGE and Western Blot Analysis

Met-5A and NCI-H28 cells were grown in 150 mm Petri dishes, cultured until confluence and then serum-starved overnight at 37°C. After incubation, cells were washed twice with ice-cold PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 137 mM NaCl and 2.7 mM KCl) and lysed in modified RIPA buffer (PBS, pH 7.4, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS and 10 µl/ml protease inhibitor cocktail). Cells were lysed for 1 h at 4°C, then were centrifuged at 14 000 x g in a pre-cooled centrifuge for 45 min. The supernatant was immediately transferred to a fresh centrifuge tube and the pellet was discarded. To measure the total protein content, the Bio-Rad DC protein assay was used following the manufacturer's instructions. BSA was used as standard.

Solubilized proteins (30 µg) of each sample (Met-5A and NCI-H28) were treated with Laemmli solution, and then resolved by 12% SDS-PAGE gels using 20 mA for gel (Tetra cell apparatus, Biorad). Proteins were then transferred onto nitrocellulose membranes (0.2 µm) using 100 V for 30min. Non-specific binding was prevented by blocking the membranes with 3% low fat dried milk, 0.2% (v/v) Tween-20 in PBS (10 mM NaH_2PO_4, pH 7.4, 0.9% NaCl) (PBS/milk/Tween) for 1 h at room temperature. Subsequentially, nitrocelluloses were probed overnight at 4°C in blocking buffer with a rabbit anti-PAR_1 polyclonal antibody (1:200) or a rabbit anti-PAR_2 (1:200) polyclonal antibody. Following three 5 minutes washes with PBS/milk/Tween, the immunocomplexes were detected using a peroxidase-labelled secondary antibody (goat anti-rabbit, 1:10 000 dilution for PAR_1 and 1:20 000 dilution for PAR_2). The washing step was repeated as described above, followed by two washes with PBS and one with distilled water. The immunoblot signal was visualized by using enhanced chemiluminescence substrate detection system (Western Lightning® Plus-ECL). The chemiluminescent images were acquired by LAS4010 (GE Health Care). The intensity of

immunoreactive bands was estimated from optical density measurements of scanned images using Image J (NIH, Bethesda, MD), a public domain JAVA image-processing program. Band optical density values were normalized by the value of total protein loaded on each well.

Each nitrocellulose membrane probed with anti-PAR_1 or anti-PAR_2 antibodies was stripped and re-probed with a mouse anti-β-actin monoclonal antibody (1:2000) for 2 h at room temperature followed by an anti-mouse peroxidase-labelled secondary antibody (1:2000).

2.5 WST-1 cell proliferation assay

The assay was based on the enzymatic cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (from Roche) to colored formazan by cellular mitochondrial dehydrogenases present in viable cells.

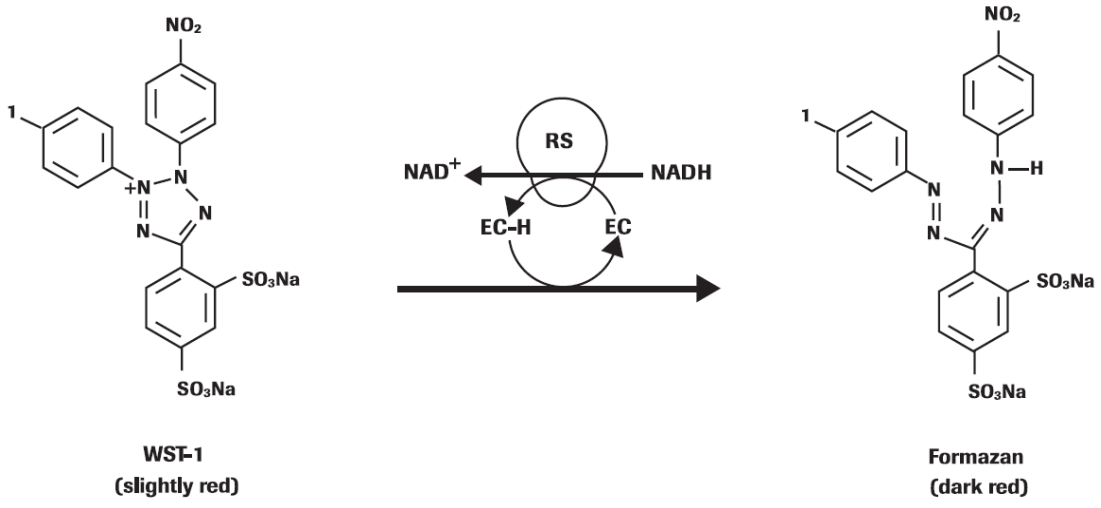


Figure. Cleavage of the tetrazolium salt WST-1 to formazan. (EC = electron coupling reagent. RS = mitochondrial succinate-tetrazolium-reductase system).

An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture.

Cells were seeded in a clear 96-well plate at a density of 3 x 10^3 cells/well in 100 µl of culture medium. Cells were cultured in the incubator at 37°C and allowed to adhere overnight, then cells were serum starved for 12 hours and stimulated with the agonist for 24 or 72 hours. After that, 10 µl of WST-1 mixture were added to each well, mixed gently for one minute on a shaker and cells were incubated for two hours at 37°C in a CO_2 incubator. Finally, the formazan dye, produced by metabolically active cells, was quantified by measuring the absorbance of each sample against a background control as blank with a Wallac 1420 multilabel counter microplate reader (PerkinElmer, Inc., Boston, MA) at a wavelength of 450 nm.

2.6 $[Ca^{2+}]_i$ measurement

PAR_1-mediated activation of G_q pathway was assessed by measuring the increase of fluorescence after agonist stimulation of cells loaded with the Fluo-3 acetoxy methylester (Fluo-3-AM), using a Wallac 1420 multilabel counter microplate reader (PerkinElmer, Inc., Boston, MA). Fluo-3 exhibits large fluorescence intensity increases on Ca^{2+} binding.

Cells were seeded in black/clear bottom 96-well assay plates at a density of 2 x 10^4 cells/well (HMEC-1) or 1.5 x 10^4 cells/well (Met-5A and NCI-H28) in complete growth medium. After attachment they were starved in serum free medium containing bovine serum albumin (BSA) for 3 h at 37°C. Before starting the assay, cells were washed twice with

loading buffer (20 mM Hepes, 0.83 mM Na_2HPO_4, 0.17 mM NaH_2PO_4, pH 7.4, + 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgSO_4$) containing 25 mM mannose, 1 mg/ml BSA and 2.5 mM probenecid and then incubated in 100 µl of the same buffer containing 6 µM Fluo-3-AM/0.024% pluronic acid. The fluorescent probe can penetrate the cells by the presence of the ester, which is subsequently hydrolyzed by intracellular esterases to an active form. The presence of probenecid further reduce any possible leak of Fluo-3 from the cells, since it is a standard inhibitor of nonspecific organic anion carrier. After 1 h at 37°C, cells were washed two times with loading buffer and incubated in 100 µl of the same buffer for an additional 1 h at 37°C to remove any excess of dye. Fluorescence measurements, reflecting the increase of $[Ca^{2+}]_i$, were carried out at 37°C with an excitation wavelength of 480 nm and emission recorded at 530 nm. Fluorescence was recorded at baseline and then every 3 seconds after thrombin (50 nM) or PAR-AP (10 µM) addition for another 120 seconds. The data were expressed as fluorescence ratios.

Whereas the selective PAR_1- and PAR_2-APs were purchased from Tocris as mentioned in Materials the nonselctive PAR_1-AP was synthesized in Dr. A.M. D'Ursi's laboratory (Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno).

PAR-AP sequences

Peptides	**Sequence**
Selective PAR_1-AP (Tocris)	TFLLRN-NH_2
Selective PAR_2-AP (Tocris)	2-furoyl-LIGRLO-NH_2
PAR_1-AP	SFLLRN-NH_2

2.7 RhoA activation

Cells were seeded in 150 mm dishes until 60% confluence and serum-starved for 18 hours. Cells were then stimulated with 10 nM thrombin for zero and two minutes. After that, culture dishes were placed on ice and washed with ice cold PBS. Then, cells were lysed with 500 µl ice-cold cell lysis buffer supplemented with protease inhibitors. Lysates were transferred into a tube and centrifuged at 10,000 g for 3 minutes at 4°C. Afterwards, cell lysates were added to a 96 well plate (RhoA G-LISA® kit from Cytoskeleton) that contained a Rho-GTP-binding protein linked to the wells. Active, GTP-bound Rho in cell lysates bound to the wells while inactive GDP-bound Rho was removed during washing steps. The bound active RhoA was detected with a RhoA specific antibody. The degree of RhoA activation was determined by comparing readings from activated cell lysates versus non-activated cell lysates. Inactivation of RhoA was generally achieved in cell culture by the serum starvation step. Finally, the absorbance of each sample was measured by using a Wallac 1420 multilabel counter microplate reader (PerkinElmer, Inc., Boston, MA) at a wavelength of 490 nm and results were expressed as absorbance ratios.

2.8 Measurement of intracellular cAMP

Intracellular cAMP levels were measured using a competitive protein binding method. Cells (4 x 10^4/well) were plated in 24-well plates in 0.5 ml of medium. After 24 h, the medium was removed and 0.5 ml of serum free medium with phosphodiesterase inhibitor, Ro 20-1724 (20 µM), was added and the cells incubated at 37°C for 15 min. Cells were then exposed to different thrombin and peptide concentrations ranging from 100 nM to 10 µM (15

min at 37°C). Assays were initiated by the addition of 1 µM isoproterenol. The reaction was terminated by the removal of the medium and the addition of 0.4 N HCl. After 30 min, lysates were neutralized with 4 N KOH, and the suspension centrifuged at 800 x g for 5 minutes. To measure cAMP production, bovine adrenal cAMP binding protein was incubated with [^{3}H]cAMP (2 nM) and 50 µl of cell lysates or cAMP standard at 0°C for 150 min in a total volume of 300 µl. Bound radioactivity was separated by rapid filtration through GF/C glass fiber filter using a cell harvester. The radioactivity was measured by liquid scintillation spectrometry.

2.9 Data Analysis

Data analysis were performed by the computer program GraphPad Prism for Windows (GraphPad Software, San Diego, CA). Values represent the means ± S.E.M. of at least three independent experiments. The statistical significance of value differences was evaluated by ANOVA test followed by Bonferroni's multiple comparison test using GraphPad Prism for Windows.

RESULTS

PARs and their potential activating proteases are frequently overexpressed in human tumor tissues, including prostate and colon cancer as well as malignant melanoma (Arora *et al.*, 2007; Bar-Shavit *et al.*, 2011; Ramachandran *et al.*, 2012). Lee *et al.* (2005) have shown that PAR_2 is present in human pleural tissues where it plays a role in pleural inflammatory responses while in primary cultures of human peritoneal mesothelial cells the expression of PAR_1 has been reported (Belling *et al.*, 2013). Therefore, we decided to investigate expression and signaling of PARs in human pleural mesothelial and MM cells to evaluate the role of these receptors in mesothelioma cell proliferation and survival. As first step of this study, the MM cell line NCI-H28 which does not express CXCR4 (Li *et al.*, 2011), was used while the nonmalignant mesothelial cell line Met-5A was utilized as control. In this MM cell line, apart from a homozygous deletion of the β-catenin gene (CTNNB1) (Shigemitsu *et al.*, 2001) a down-regulation of thrombomodulin expression by an epigenetic mechanism has been described (Nocchi *et al.*, 2011). The expression of thrombomodulin, a glycosilated transmembrane protein which binds with high affinity thrombin inhibiting its enzymatic activity and accelerating protein C activation, is lower in MM tissue than in normal mesothelium (Nocchi *et al.*, 2011). In addition, low or no expression of the protein in various cancers has been associated with poor prognosis (Matsushita *et al.*, 1998; Ogawa *et al.*, 2000; Liu *et al.*, 2010).

To verify whether PAR_1 and PAR_2 mRNA levels were different in NCI-H28 cells as compared to those in Met-5A cells, real time RT-PCR was performed using RNA extracted from MM and nonmalignant control cells, respectively. In NCI-H28 cells, PAR_1 mRNA level was approximately 3-fold higher than in Met-5A cells while PAR_2 mRNA amount was slightly reduced in MM cells as compared to nonmalignat control cells (Fig. 1, panel A). Similarly, immunoblot analysis showed that in NCI-H28 cells the expression level of PAR_1 was significantly increased as compared to Met-5A cells while PAR_2 expression was

substantially unchanged in MM cells (Fig. 1, panel B). These results indicate that the increased expression of PAR_1 in NCI-H28 is mainly dependent by an increase of gene transcription and/or modification of PAR_1 mRNA stability.

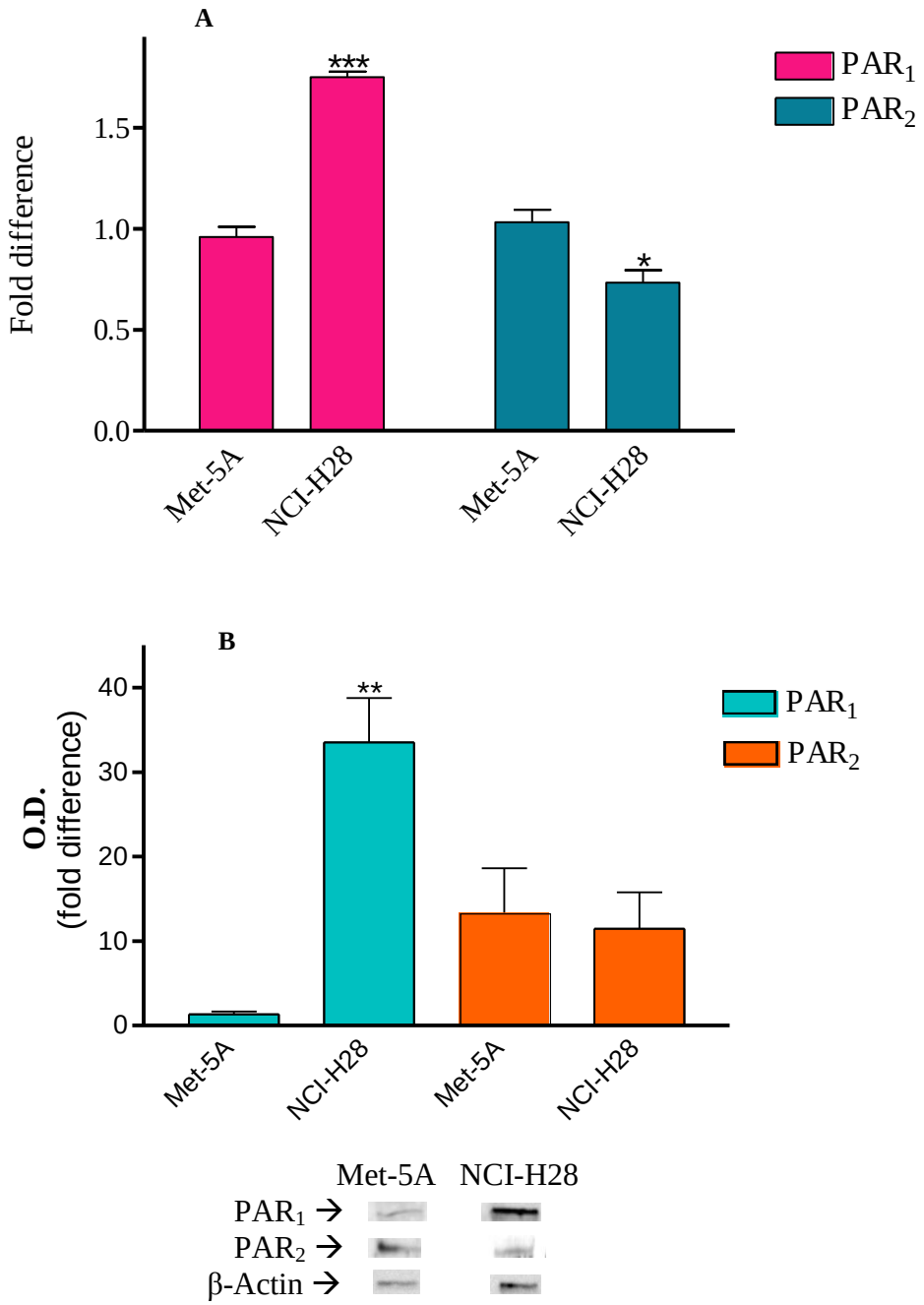


Figure 1. Detection of PAR mRNA (panel A) and protein levels (panel B) by real time RT-PCR and western blot analysis, respectively, in Met-5A and NCI-H28 cells. Data are normalized to β-actin. Data shown are mean ± SEM of at least three independent experiments. Values that are significantly different from control value (* $p<0.05$; ** $p<0.01$; *** $p<0.001$), as determined by ANOVA followed by Bonferroni's multiple comparison test, are indicated.

Next, we examined whether in the MM cell line PAR_1 was functionally active by evaluating thrombin- or PAR-APs-induced cell proliferation. After 72 h from thrombin stimulation, both Met-5A and NCI-H28 cells showed significant increase of cell proliferation (Fig. 2). However, the pattern of the proliferative response was quite different in MM cells as compared to that of nonmalignant mesothelial cells. In fact, in Met-5A the proliferative response was maximal at 1 nM thrombin with a progressive decrease up to 50 nM while in NCI-H28 cells the maximal response was reached at 50 nM (Fig. 2).

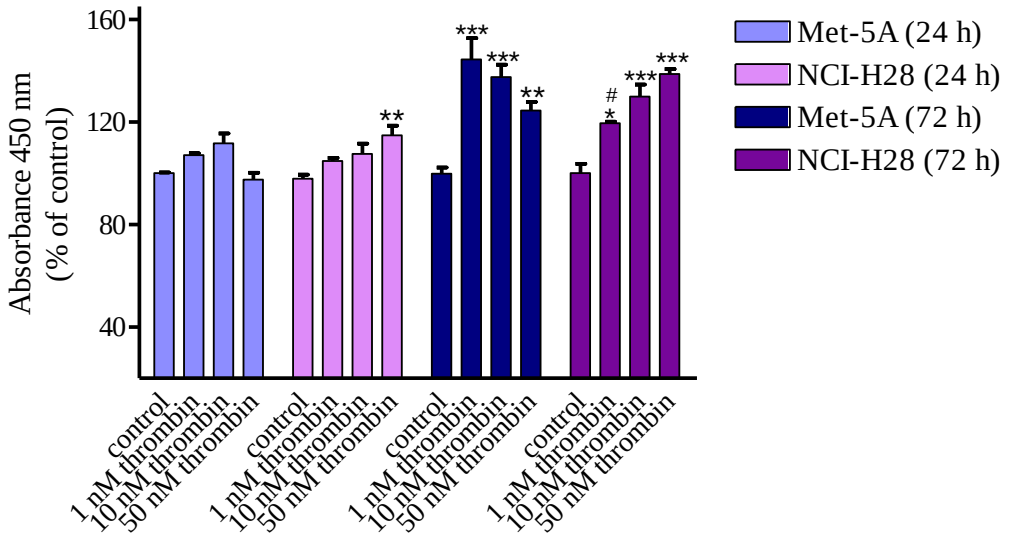
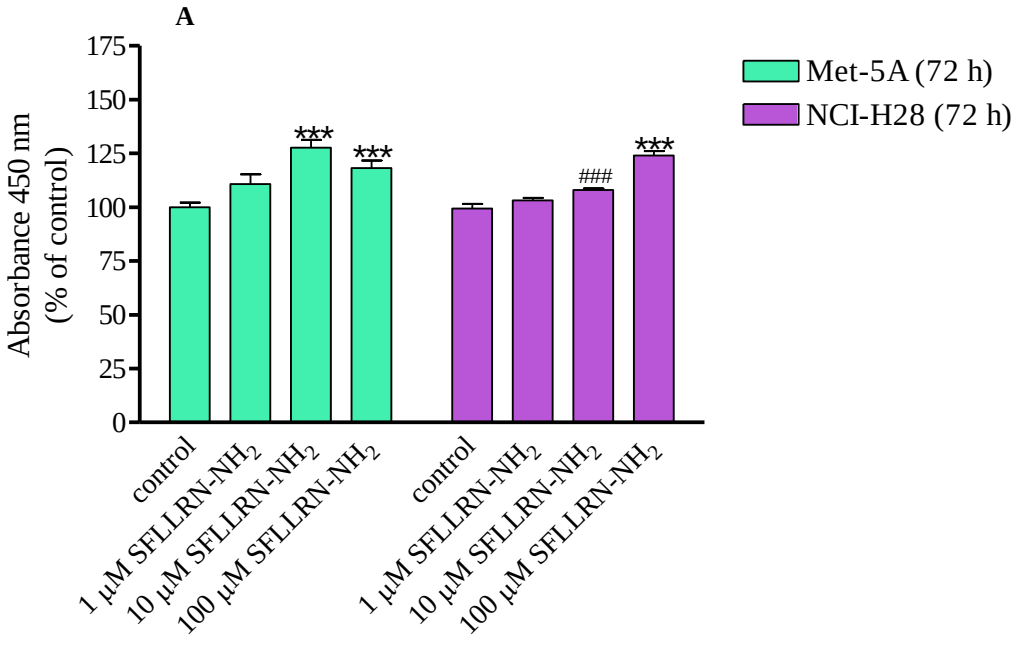


Figure 2. Thrombin-induced proliferation of Met-5A and NCI-H28 cells. Cells were seeded at a density of 3 x 10^3 cells/well and stimulated with the agonist for 24 or 72 hours. Data shown are mean ± SEM of at least three independent experiments. Values that are significantly different from control value (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) and from the reference value in Met-5A ($^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$; $^{\#\#\#}$ $p<0.001$), as determined by ANOVA followed by Bonferroni's multiple comparison test, are indicated.

The non-selective PAR_1-AP, SFLLRN-NH_2, was less effective than thrombin in stimulating Met-5A and NCI-H28 cell proliferation (Fig. 3, panel A). A 24 – 28% increase of cell proliferation was reached at 10 and 100 µM SFLLRN-NH_2 in Met-5A and NCI-H28 cells, respectively (Fig. 3, panel A). The selective PAR_1-AP, TFLLR-NH_2, was less effective

in stimulating Met-5A cell proliferation than SFLLRN-NH_2 but a concentration of 100 µM caused a 20% increase of NCI-H28 cell proliferation (Fig. 3, panel B). To evaluate the role of PAR_2 in stimulating mesothelial cell proliferation, both cell lines were stimulated with a selective PAR_2-AP, 2-furoyl-LIGRLO-NH_2. Treatment of Met-5A cells with the selective PAR_2-AP caused inhibition of cell proliferation while in NCI-H28 cells 10 and 100 µM 2-furoyl-LIGRLO-NH_2 induced approximately 20% increase of cell proliferation (Fig. 3, panel C). These results point out that PAR-APs do not behave as thrombin in activating PAR signaling. Furthermore, both PAR_1 and PAR_2 signaling appears to be quite different in NCI-H28 as compared to receptor signaling in Met-5A cells.



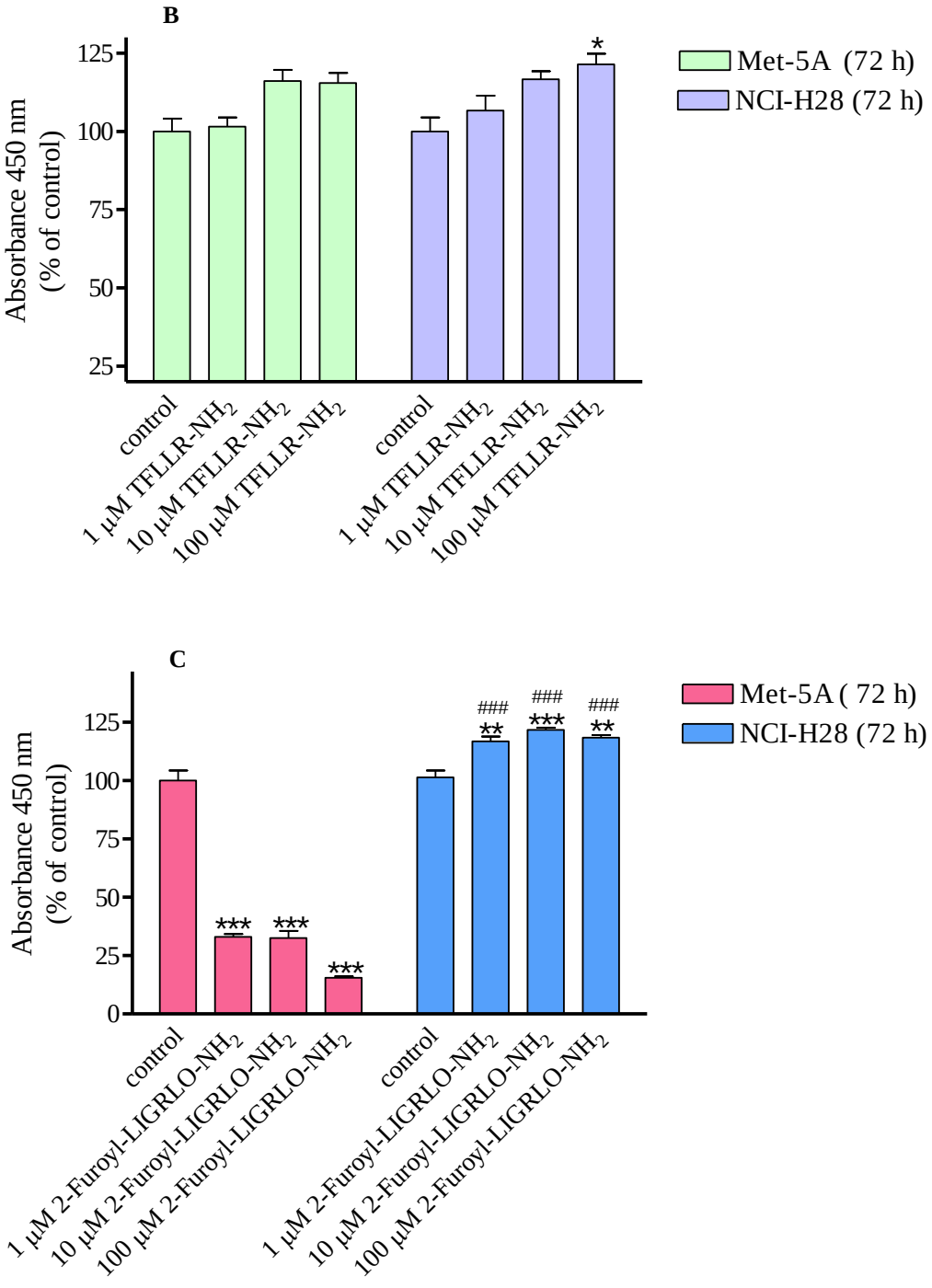
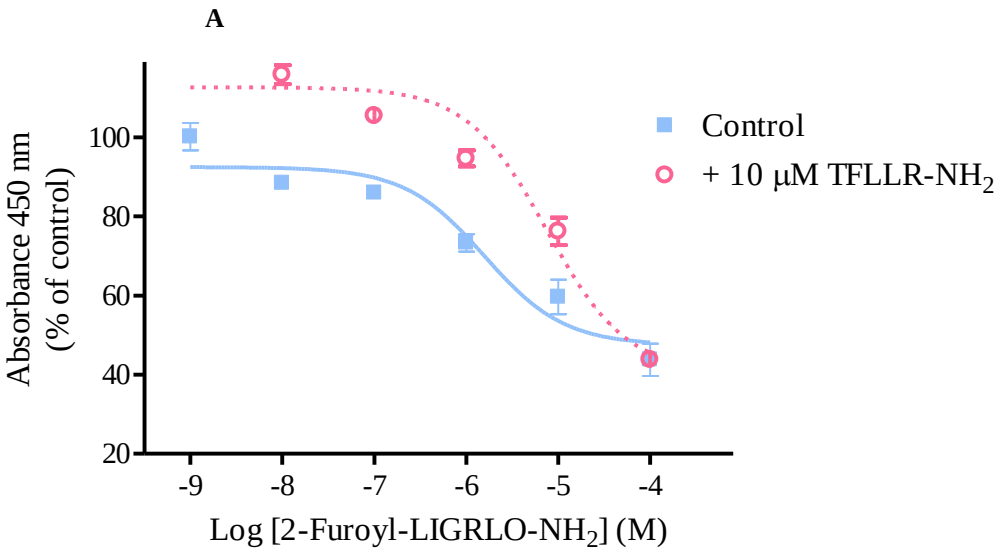


Figure 3. Non-selective PAR_1-AP- (panel A), selective PAR_1-AP- (panel B) and selective PAR_2-AP-induced (panel C) proliferation of Met-5A and NCI-H28 cells. Cells were seeded at a density of 3 x 10^3 cells/well and stimulated with the agonist for 72 hours. Data shown are mean ± SEM of at least three independent experiments. Values that are significantly different from control value (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) and from the reference value in Met-5A ($^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$; $^{\#\#\#}$ $p<0.001$), as determined by ANOVA followed by Bonferroni's multiple comparison test, are indicated.

In order to understand whether thrombin- or SFLLRN-NH_2-activated PAR_1 is able to cross-talk and activate PAR_2 as our results seem to suggest, we performed additional proliferation experiments using selective PAR-APs in combination, thrombin in the presence and absence of a PAR_1 or PAR_2 antagonist, or the non selective SFLLRN-NH_2 peptide in the presence and absence of the PAR_1 antagonist. The selective PAR_2 peptide inhibited in a concentration-dependent manner Met-5A cell proliferation reaching a 56% inhibition at 100 µM. In the presence of 10 µM TFLLR-NH_2, the inhibition curve of 2-furoyl-LIGRLO-NH_2 was upwards and right shifted but reached the maximal effect (56% inhibition) at 100 µM (Fig. 4, panel A). On the other hand, TFLLR-NH_2 and 2-furoyl-LIGRLO-NH_2 were synergistic in stimulating NCI-H28 cell proliferation (Fig. 4, panel B).



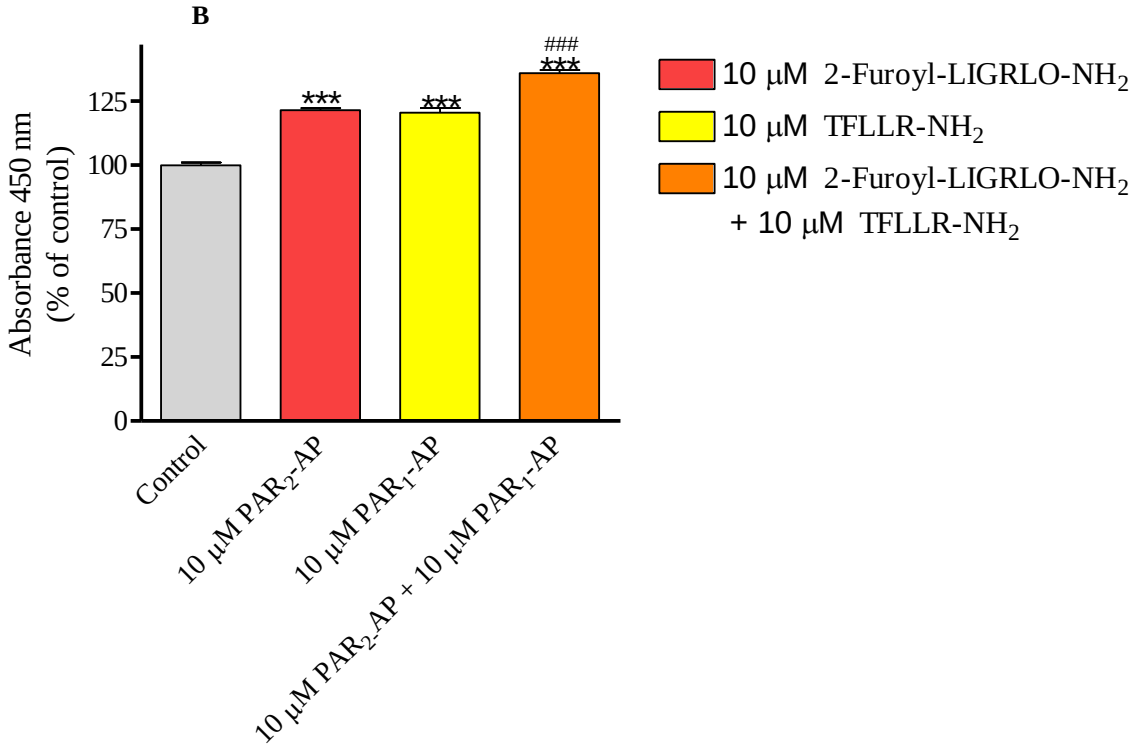


Figure 4. Effect of selective PAR_2-AP on Met-5A (panel A) and NCI-H28 (panel B) cells, in presence and absence of PAR_1-AP. Cells were seeded at a density of 3 x 10^3 cells/well and stimulated with the agonist for 72 hours. Data shown are mean ± SEM of at least three independent experiments. Values that are significantly different from control value (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) and from the value with one peptide ($^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$; $^{\#\#\#}$ $p<0.001$), as determined by ANOVA followed by Bonferroni's multiple comparison test, are indicated.

In Met-5A cells, the proliferative effect of thrombin was modestly reduced by the PAR_1 antagonist, SCH 79797 while in NCI-H28, thrombin-induced proliferation significantly decreased in the presence of the receptor antagonist (Fig. 5, panel A and B).

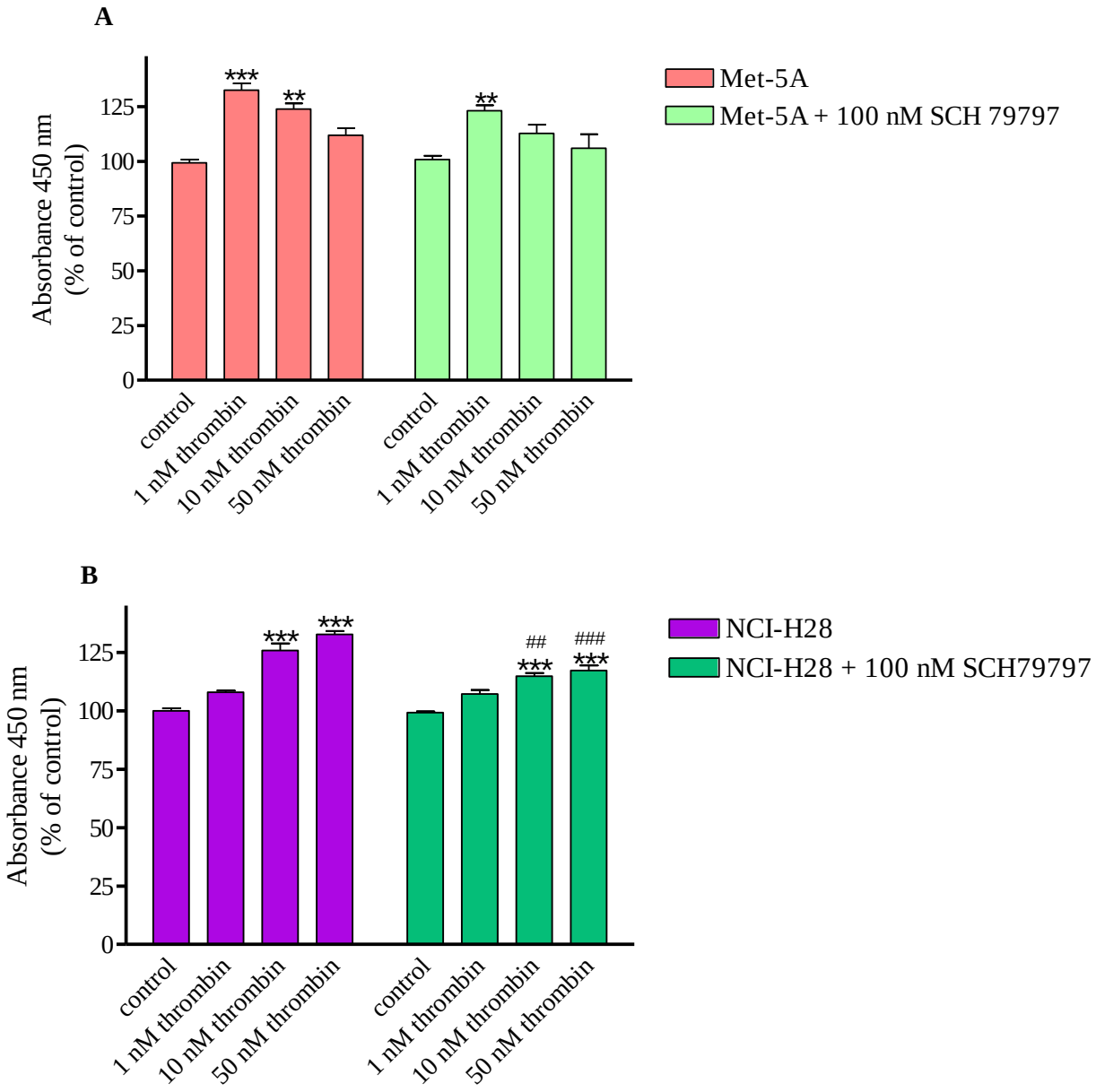


Figure 5. Thrombin induced proliferation of Met-5A (panel A) and NCI-H28 (panel B) cells, in the presence and absence of PAR_1 antagonist. Cells were seeded at a density of 3 x 10^3 cells/well and stimulated with the agonist for 72 hours. Data shown are mean ± SEM of at least three independent experiments. Values that are significantly different from control value (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) and from the reference value in Met-5A ($^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$; $^{\#\#\#}$ $p<0.001$), as determined by ANOVA followed by Bonferroni's multiple comparison test, are indicated.

Moreover, Met-5A and NCI-H28 cell proliferation induced by SFLLRN-NH_2 was only modestly reduced in the presence of SCH 79797 (Fig. 6, panel A and B) indicating that this activating peptide stimulate cell proliferation through independent activation of both PAR_1 and PAR_2.

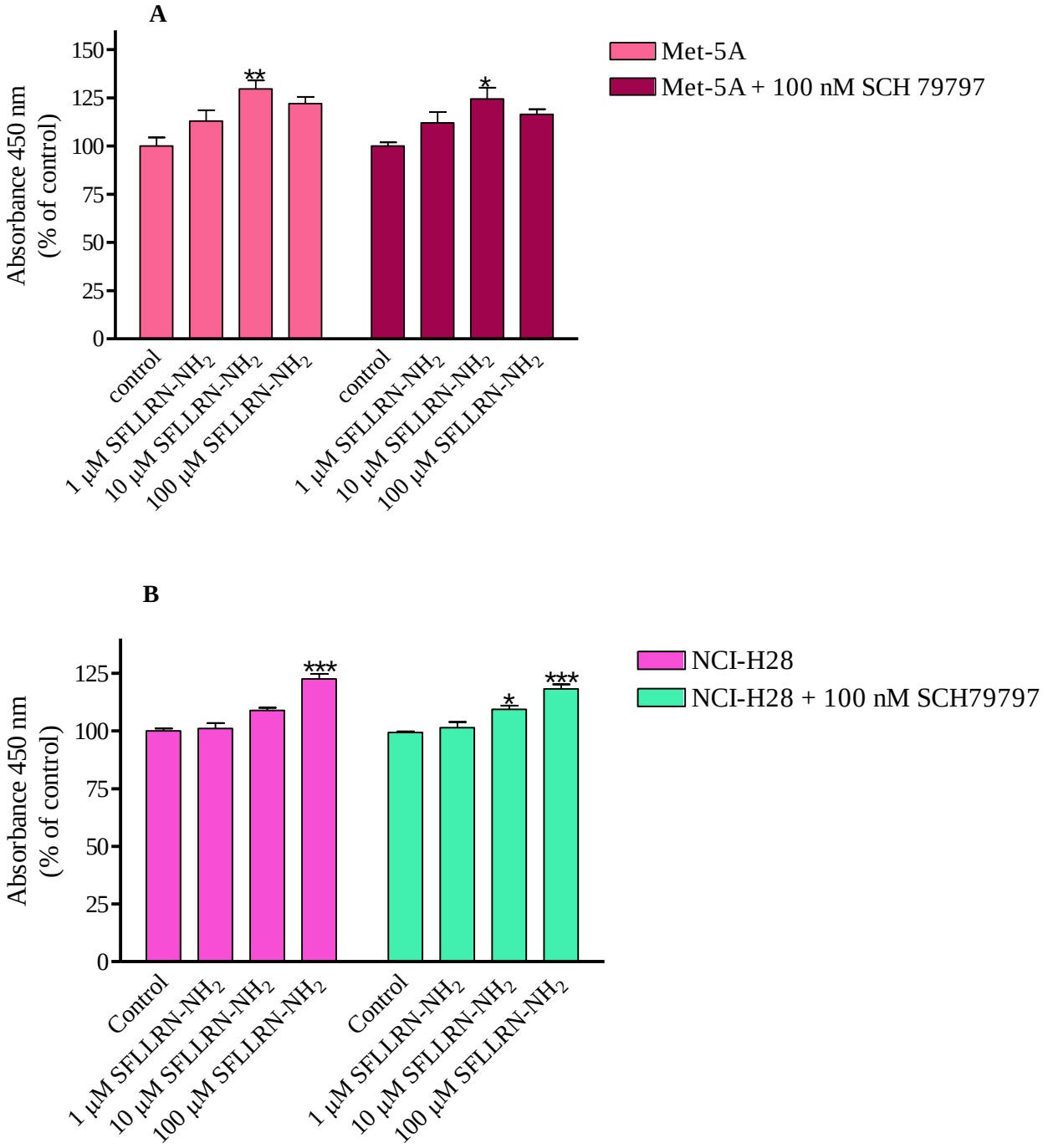


Figure 6. Non-selective PAR_1-AP induced proliferation of Met-5A (panel A) and NCI-H28 (panel B) cells, in the presence and absence of PAR_1 antagonist. Cells were seeded at a density of 3 x 10^3 cells/well and stimulated with the agonist for 72 hours. Data shown are mean ± SEM of at least three independent experiments. Values that are significantly different from control value (* $p<0.05$; ** $p<0.01$; *** $p<0.001$), as determined by ANOVA followed by Bonferroni's multiple comparison test, are indicated.

Finally, we examined the effect of the PAR_2 antagonist, GB83, on thrombin-induced cell proliferation. This antagonist modified the proliferative response of neither Met-5A nor NCI-H28 cells (Fig. 7) suggesting that PAR_1 tethered ligand does not transactivate PAR_2 in these cells. Moreover, these experimental evidences support the existence of altered PAR_1 and PAR_2 signaling in the malignant mesothelioma cell line.

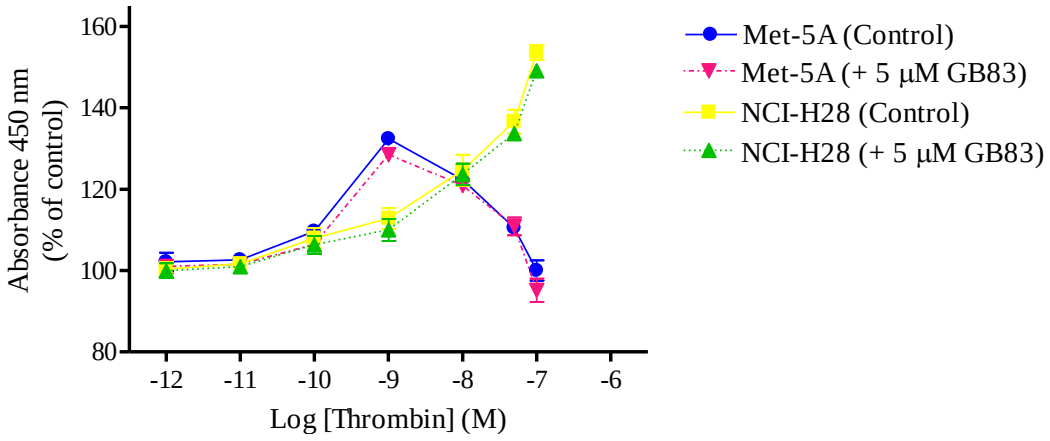


Figure 7. Thrombin-induced proliferation of Met-5A and NCI-H28 cells in the presence and absence of a PAR_2 antagonist. Cells were seeded at a density of 3 x 10^3 cells/well and stimulated with the agonist for 72 hours. Data shown are mean ± SEM of at least three independent experiments.

In order to address this question, receptor-induced G_q, $G_{12/13}$, and G_i signaling pathways were examined. First, we investigated PARs-activated G_q signaling by analyzing intracellular Ca^{2+} mobilization after cell stimulation with either thrombin or PAR-APs. To measure Ca^{2+} transient, Fluo 3-AM and a Wallac 1420 multilabel counter microplate reader were utilized as described under Material and Methods. As indicated by relative fluorescence increase, both thrombin (10 nM) and selective PAR-APs (10 µM) induced rapid and transient increase of $[Ca^{2+}]_i$ in Met-5A as well as in microvascular endothelial cells (HMEC-1) (Fig. 8, panel A and B). On the contrary, in NCI-H28 cells, thrombin- and PAR-APs-stimulation did not cause any rapid increase of $[Ca^{2+}]_i$ (Fig. 8, panel A and B). However, Met-5A and NCI-H28

showed similar expression levels of proteins involved in this signaling pathway, such as $G\alpha_q$ and phospholipase C-β (PLC-β) isoforms (data not shown).

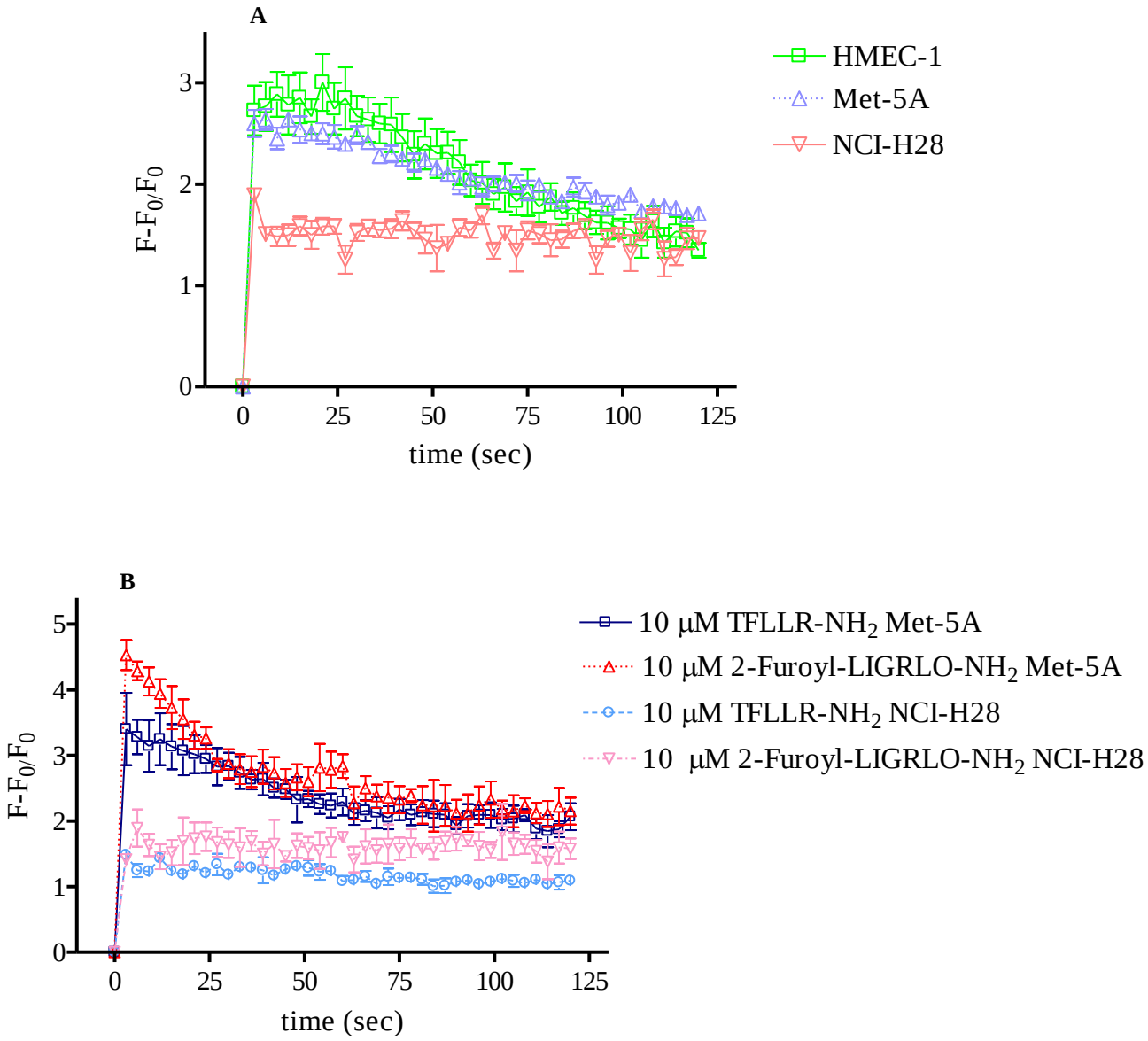
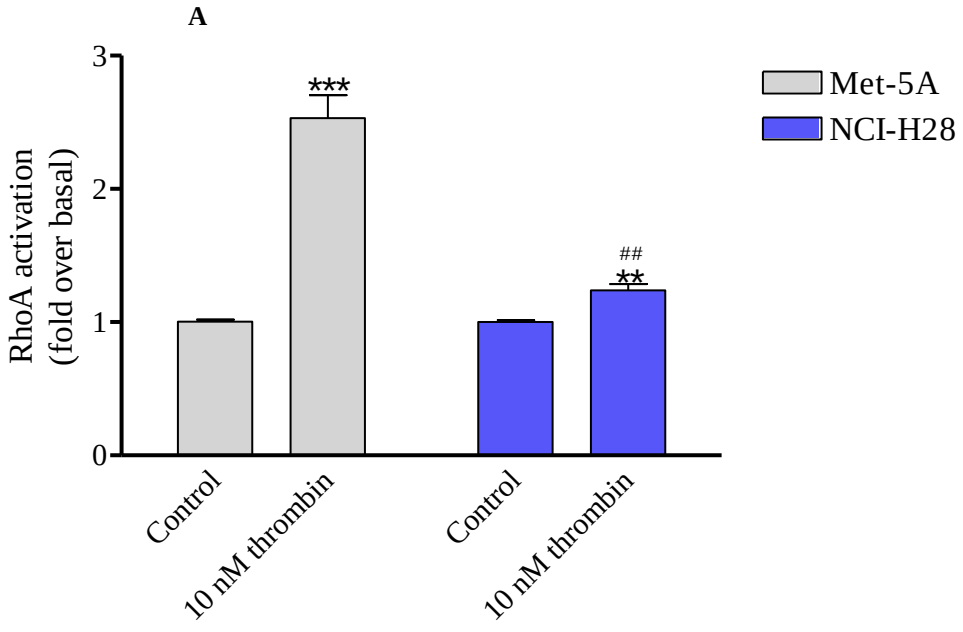


Figure 8. Intracellular Ca^{2+} mobilization induced by thrombin (panel A) and selective PAR-APs (panel B) in HMEC-1 (2 x 10^4 cells/well), Met-5A (1.5 x 10^4 cells/well) and NCI-H28 (1 x 10^4 cells/well) cells. Cells were loaded with Fluo-3AM to measure $[Ca^{2+}]_i$ variations as indicated by changes in fluorescence intensity. Fluorescence was monitored for up to 120 seconds. Data are mean ± SEM of at least three independent experiments and are reported as percent of maximal relative fluorescence (RF = $(F - F_0)/F_0$ where F_0 is basal fluorescence and F is fluorescence recorded after cell stimulation with the agonist).

Next, we examined PARs-induced $G_{12/13}$ signaling by measuring RhoA activation after cell stimulation with either thrombin or PAR-APs. To detect RhoA activation the RhoA G-LISA® kit from Cytoskeleton was used as described under Material and Methods. In Met-5A cells, 10 nM thrombin induced a significant 2.5-fold increase of RhoA activation while in NCI-H28 cells the increase was just 1.2-fold (Fig. 9, panel A). The selective PAR_1-AP (10 µM) was less effective in stimulating RhoA activation than thrombin in Met-5A cells but it still caused a significant ~2-fold increase (Fig. 9, panel B). Similarly to thrombin, PAR_1-AP induced a modest increase of RhoA activation in NCI-H28 cells (Fig. 9, panel B). Stimulation of both Met-5A and NCI-H28 cells with the selective PAR_2-AP (10 µM) did not induce any RhoA activation (Fig. 9, panel C). It is important to mention that in both cell lines the expression levels of Gα12, Gα13, and RhoA were similar (data not shown).



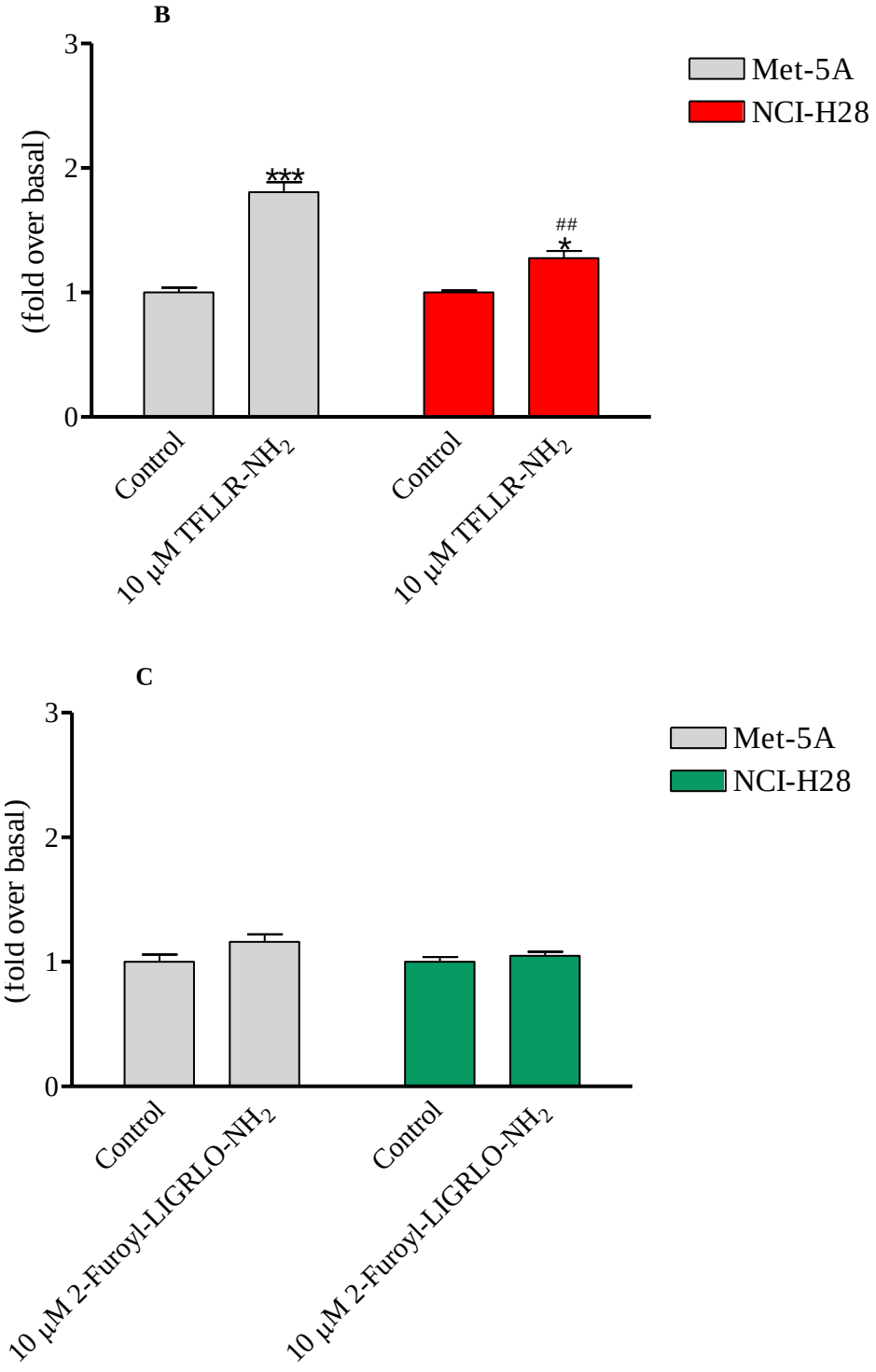


Figure 9. RhoA activation in response to thrombin (panel A), PAR_1-AP (panel B) and PAR_2-AP (panel C) in Met-5A and NCI-H28 cells. RhoA activation was measured using RhoA G-LISA® kit from Cytoskeleton. Data shown are mean ± SEM of at least three independent experiments. Values that are significantly different from control value (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) and from the reference value in Met-5A ($^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$; $^{\#\#\#}$ $p<0.001$), as determined by ANOVA followed by Bonferroni's multiple comparison test, are indicated.

Finally, thrombin- and PAR-APs-induced inhibition of isoproterenol stimulated cAMP accumulation was examined in both Met-5A and NCI-H28 cells. In Met-5A cells, 10 pM to 1 nM thrombin inhibited isoproterenol stimulated cAMP production in a concentration dependent manner reaching 50% inhibition at 1 nM (Fig. 10, panel A). However, at higher thrombin concentrations (1 nM to 100 nM) the inhibitory effect was progressively reverted. In the presence of the PAR_1 antagonist SCH 79797 the inhibitory effect of thrombin was significantly reduced indicating that PAR_1 mediates such effect. In NCI-H28 cells, thrombin inhibited cAMP in a concentration dependent manner reaching 50% and maximal inhibition (~ 70%) at 1 nM and 100 nM, respectively (Fig. 10, panel A). In the presence of SCH 79797, the inhibition curve was upwards shifted and the maximal inhibition at 100 nM was only 42%. Various concentrations of the selective PAR-APs did not cause any inhibition of isoproterenol stimulated cAMP production in both Met-5A and NCI-H28 cells demonstrating the functional selectivity of these agonists.

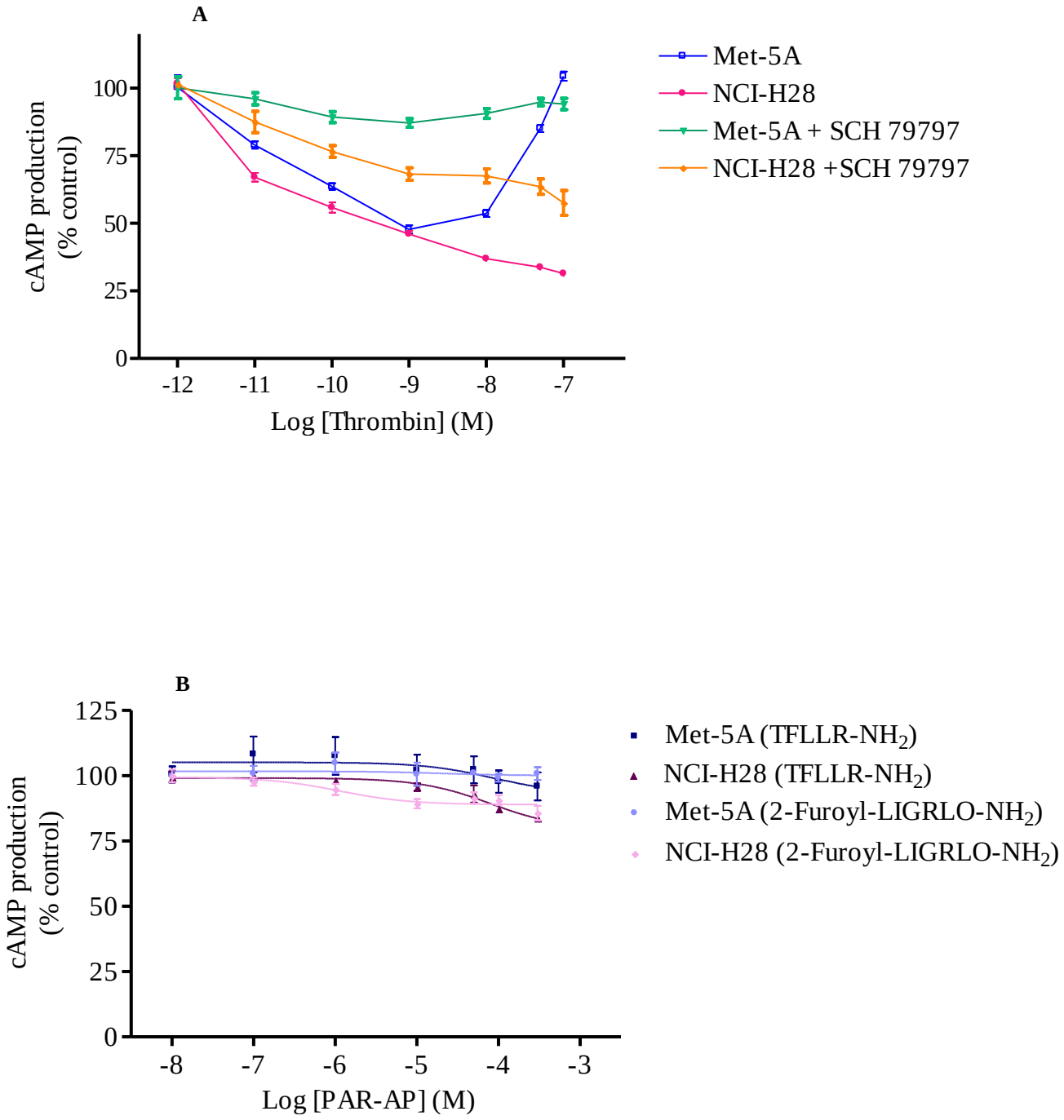


Figure 10. Inhibition of isoproterenol stimulated cAMP production by thrombin (panel A) and selective PAR-APs (panel B) in Met-5A and NCI-H28 cells Production of cAMP was measured using a competition binding assay which includes the bovine adrenal cAMP binding protein and [^{3}H]cAMP. Data shown are mean ± SEM of at least three independent experiments.

DISCUSSION AND CONCLUSIONS

PARs elicit cellular response to coagulant proteases triggered by vascular injury (Coughlin, 2005). In addition, coagulant proteases and PARs have been implicated in several types of malignant tumors. Indeed, a well-documented link between hyperactivation of the coagulation cascade and tumor progression exists. The procoagulant activity mediated by the action of coagulant proteases such as thrombin can contribute to the malignant phenotype both directly, by stimulating tumor cell proliferation, and indirectly through the development of tumor-associated thromboemboli (Nierodzik and Karpatkin, 2006). Among all cancer patients, MM patients are very susceptible to thromboembolic complications (Nguyen *et al.*, 2008). Therefore, it seemed quite important to investigate whether a correlation exists between PAR expression and proliferation in a MM cell line, NCI-H28, in which thrombomodulin, a transmembrane glycoprotein that control thrombin-mediated proteolysis, is silenced by an epigenetic mechanism (Nocchi *et al.* 2011).

Indeed, the proliferative response of NCI-H28 cells to various concentrations of thrombin is quite different than that obtained in nonmalignant mesothelial cells, Met-5A. Whereas in MM cells thrombin-induced proliferation rapidly increases at agonist concentrations ranging from 0.1 nM to 100 nM in Met-5A cells thrombin induces the maximal effect at 1 nM and then at higher concentrations the stimulatory effect progressively decrease reaching at 100 nM the basal level (Fig. 5). Although NCI-H28 cells express higher amounts of $PAR_{1,}$ receptors respond to higher thrombin concentrations with the same level of proliferation than nonmalignant mesothelial cells. However, in MM cells the proliferative response increases without reaching any growth steady state as expected when cells lose contact inhibition which is a typical characteristic of cancer cells. In MM cells, the observed differences can result from alteration of the cross-talk of PAR_1 with other proteins, including PAR_2, or by the absence of thrombomodulin on cell membrane. We addressed the former issue by examining PAR-APs- and thrombin-induced proliferation in the presence and

absence of antagonists. The experiments with a non selective peptide, SFLLRN-NH_2, which activate both PAR_1 and PAR_2 (Ramachandran *et al.*, 2012) demonstrate that both receptors are able to induce a proliferative response in nonmalignant mesothelial and MM cells. In NCI-H28 cells, selective PAR_1- and PAR_2-APs show a synergistic effect but the same does not occur in Met-5A cells suggesting differences in the mechanism of receptor activation and/or signaling. At this moment we cannot say whether in MM cells PAR_2 is mutated and thus activation and signaling are altered. Since the nonselective peptide stimulates proliferation in Met-5A as well as in NCI-H28, we are inclined towards believing the inhibitory effect of 2-furoyl-LIGRLO-NH_2 on Met-5A cell proliferation as a consequence of functional selectivity. This is a process reported for many GPCRs including PAR_1 (McLaughlin *et al.*, 2005; Russo *et al.*, 2009) by which different ligands acting at the same receptor can elicit distinct signaling responses. The molecular basis of functional selectivity involves ligand induced stabilization of distinct active GPCR conformation (Urban *et al*, 2007). Moreover, compartmentalization of GPCRs within membrane microdomains seems to facilitate stabilization of distinct active conformations promoting receptor coupling to specific G proteins and therefore signaling effectors (Zheng *et al.*, 2008).

O'Brien *et al.* (2000) reported that thrombin-cleaved PAR_1 transactivates PAR_2 in human umbilical vein endothelial cells (HUVECs) thus limiting the effectiveness of PAR_1 antagonists. We can exclude such event in both nonmalignant mesothelial and MM cells since the presence of a PAR_2 antagonist does not modify thrombin concentration-response curves. However, we do not feel to exclude the formation of heterodimeric complexes between PAR_1 and PAR_2 or even PAR_3. In both cell lines, PAR_3 expression was not tested while PAR_4 was not detectable by immunoblot analysis (data not shown). The role of thrombomodulin in modulating thrombin-induced proliferative response is still an open question which will be addressed in future experiments of thrombomodulin silencing.

When PAR_1-mediated activation of the single signaling pathways was examined, we immediately noticed that G_q and $G_{12/13}$ signaling was compromised in NCI-H28 cells. In addition, the selective PAR_2-AP failed to mobilized intracellular Ca^{2+} in NCI-H28 cells while Met-5A cells respond with a 4-fold increase of relative fluorescence to the agonist, indicating that PAR_2-mediated activation of G_q is also compromised. Of course, we analyzed the expression levels of various proteins involved in these signaling pathways but all proteins examined showed similar levels in both cell lines (data not shown). In NCI-H28 cells, the only signaling pathway which is fully activated by PAR_1 is that through G_i proteins, leading to inhibition of adenylyl cyclase. It is interesting to note that selective PAR-APs do not cause any major inhibition of cAMP accumulation. These findings are in agreement with thrombin and PAR_1-AP displaying functional selectivity at PAR_1 as reported by McLaughlin *et al.* (2005) but they also suggest that the selective PAR_2-AP, 2-furoyl-LIGRLO-NH_2, stabilizes PAR_2 in a conformation which leads to prevalent activation of G_q in nonmalignant mesothelial cells.

The altered G_q and $G_{12/13}$ signaling with the prevalence of G_i signaling in NCI-H28 cells can explain the altered proliferative response to thrombin stimulation. Indeed, PAR_1-mediated activation of extracellular signal-regulated protein kinases 1,2 (ERK1,2) occurs through both G_q and G_i signaling (Trejo *et al.,* 1996) with consequent activation of mitogenesis. However, G_q signaling can be also involved in apoptosis induction and progression through IP_3-evoked Ca^{2+} increase and signaling (Decrock *et al.,* 2013). This can explain the biphasic proliferation curve obtained after Met-5A stimulation with various thrombin concentrations. On the other hand, the selective PAR_2-AP which causes a prevalent activation of G_q in these cells induces a profound proliferation inhibition. In the MM cell line, the same peptide stimulates proliferation probably through β-arrestin-mediated activation of ERK1/2 signaling (Arora *et al.,* 2007). Thus, the altered signaling through G_q does not

only involve PAR_1 but also PAR_2 suggesting that PARs-$G\alpha_q$ coupling is compromised in NCI-H28 cells. Whereas PAR_1-$G_{12/13}$ coupling is also dysfunctional in this cell line we cannot say much about PAR_2- $G_{12/13}$ coupling since the selective PAR_2-AP does not induce any RhoA activation in the control cell line.

The compartmentalization of PARs and G proteins in plasma membrane lipid raft microdomains such as caveolae may also confer PAR-G protein selectivity (Arora *et al.*, 2007). For example, Russo *et al.* (2009) showed the critical role for caveole in activated protein C (APC), but not thrombin, activation of PAR_1 signaling in endothelial cells. Furthermore, for other GPCRs several reports indicate that caveolin-1 is required to prolong G_q signaling (Calizo and Scarlata, 2012) and inhibits receptor coupling to $G_{i/o}$ proteins (Sato *et al.,* 2012). In thrombin stimulated endothelial cells, caveolin-1 opens cell junction by targeting catenins (Kronstein *et al.,* 2012). The recruitment of caveolin-1 to cell junctions is greatly facilitates by the presence of β-catenin in the VE-cadherin/catenin complex. In NCI-H28 cells, a homozygous deletion of the β-catenin gene (CTNNB1) has been demonstrated (Shigemitsu *et al.,* 2001) suggesting that in these cells caveolin-1 is not completely associated to cell membranes. Indeed, preliminary immunofluorescence experiments show that in MM cells caveolin-1 is partially localized in the cytoplasm while in nonmalignant mesothelial cells it colocalizes with β-cateninin at plasma membrane of cell junctions. Moreover, in NCI-28 cells, PAR_1 seems to be partially localized in the cytoplasm. Caveolin-1 may be required for proper membrane localization and G protein coupling of PAR_1.

In conclusion, we demonstrated that thrombin and PAR_1-APs display functional selectivity at PAR_1. A prevalent selectivity of the PAR_2-AP for G_q signaling was also showed for the first time. Finally, we reported numerous evidences indicating a dysfunctional PAR_1 signaling in NCI-H28 cells which results as final consequence of the lack of β-cateninin.

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